AD_____

Award Number: DAMD17-02-1-0670

TITLE: Cell Growth and Survival in Ovarian Epithelial Cancer
Project 1: AKT2 Oncogene and Its Associated Protein, APBP, in Human Ovarian Cancer

PRINCIPAL INVESTIGATOR: Santo V. Nicosia, M.D.
Jin Q. Cheng, Ph.D., M.D.

CONTRACTING ORGANIZATION: University of South Florida
Tampa, Florida 33620

REPORT DATE: August 2003

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2003	Annual (8 Jul 2002 - 7 Jul 2003)

4. TITLE AND SUBTITLE
Cell Growth and Survival in Ovarian Epithelial Cancer Project 1: AKT2 Oncogene and Its Associated Protein, APBP, in Human Ovarian Cancer

5. FUNDING NUMBERS
DAMD17-02-1-0670

6. AUTHOR(S)
Santo V. Nicosia, M.D.
Jin Q. Cheng, Ph.D., M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of South Florida
Tampa, Florida 33620

E-Mail: snicosia@hsc.usf.edu; jcheng@hsc.usf.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

During past year, we have demonstrated that AKT2 contributes to chemoresistance. The mechanism by which AKT@ induces cisplatin resistance is that AKT2 phosphorylates apoptosis signal-regulating kinase 1 (ASK1) and inhibits its kinase activity leading to inactivation of cisplatin-stimulated JNK and p38 kinase as well as Bax conformational change. Moreover, geranylgeranyltransferase I and farnesyltransferase inhibitors (GGTI and FTI) sensitize ovarian cancer cells to cisplatin-induced apoptosis.

14. SUBJECT TERMS
AKT2, genetic alteration, apoptosis, JNK/p38, cisplatin, chemoresistance

15. NUMBER OF PAGES
68

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

Three tasks have been proposed in this project: 1) Examine the clinical significance and the role of AKT2 activation/overexpression in ovarian cancer *in vitro* and *in vivo*; 2) Define the role of AKT2 interacting protein APBP in AKT2 signaling and OSE transformation; and 3) Examine AKT2 in chemoresistance and the effects of combination of FTIs and cisplatin or paclitaxel on ovarian cancer, especially drug-resistance ovarian cancer cell growth.

Body:

During the last budget year, we have determined biological significance of activation/overexpression of PI3K/AKT2 pathway in human ovarian cancer and examined the PI3K and AKT2 as targets for ovarian cancer intervention.

1. PI3K/Akt activation contributes to chemoresistance.

We have previously shown a significant increase of Akt kinase activity and AKT2 protein levels in primary ovarian carcinomas (1, 2). To determine the biological significance of AKT2 alterations in human ovarian cancer, cisplatin sensitive (A2780S and OV2008) and resistance (A2780CP and C13) ovarian cancer cell lines were stably transfected with DN-AKT2 and constitutively active AKT2 (Myr-AKT2), respectively. Cell growth and Tunel assay analyses revealed that Myr-AKT2 transfected A2780S and OV2008 became resistant to cisplatin, whereas DN-AKT2 sensitized A2780CP and C13 cells to cisplatin-inhibited tumor cell growth (Fig. 1 and data not shown).

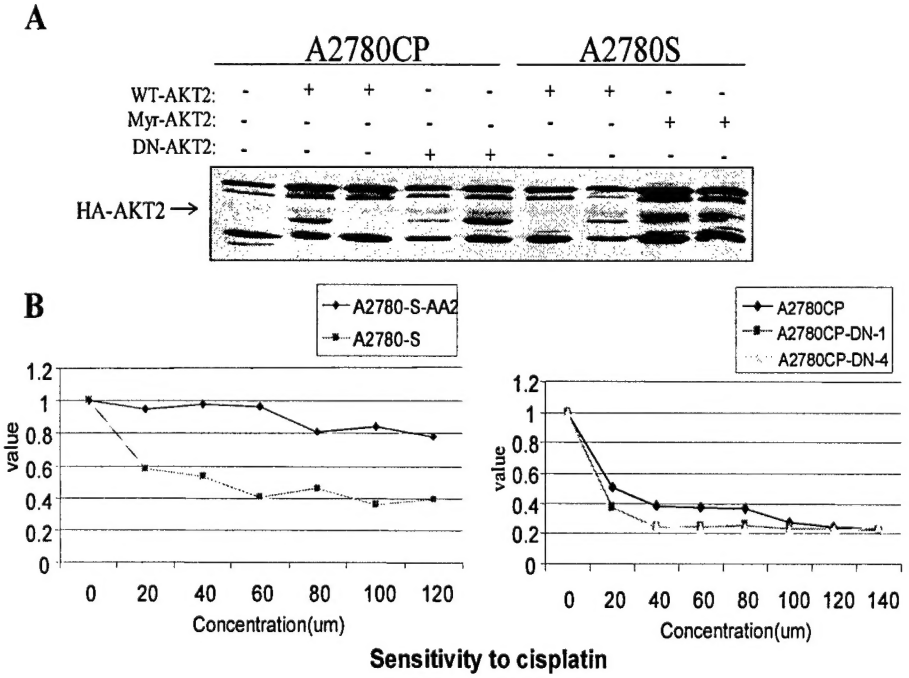


Fig. 1. *Activation of AKT2 in ovarian cancer cells contributes to chemoresistance. **(A)** Western blotting analyses of AKT2 expression. Cisplatin resistance (A2780CP) and sensitive (A2780S) cell lines were stably transfected with HA-tagged dominant negative (DN) AKT2 and constitutively active AKT2 (Myr-AKT2), respectively. Cell lysates from clonal cell lines were subjected to immunoblotting analyses with anti-HA antibody. **(B)** Clonal cell lines were treated with indicated concentration of cisplatin for 36 h. Cell viability was examined with MTS assay. Constitutively active AKT2 (AA2) transfected A2780S became resistance to cisplatin (left), whereas dominant negative AKT2 transfected A2780CP cells (both clone 1 and 4 cell lines) sensitize to cisplatin treatment as compared to parental cells (right).*

2. AKT2 inhibition of cisplatin-induced JNK/p38 activation and Bax conformational change by phosphorylation of ASK1.

Cisplatin and its analogues have been widely used for treatment of human ovarian cancer. However, most patients eventually develop resistance to treatment through a mechanism that remains obscure. Previously, we found that AKT2 is frequently overexpressed and/or activated in human ovarian and breast cancers (3). Here we demonstrate that constitutively active AKT2 renders cisplatin-sensitive A2780S ovarian cancer cells resistant to cisplatin, whereas phosphatidylinositol 3-kinase inhibitor or dominant negative AKT2 sensitizes A2780S and cisplatin-resistant A2780CP cells to cisplatin-induced apoptosis through regulation of the ASK1/JNK/p38 pathway. AKT2 interacts with and phosphorylates ASK1 at Ser-83 resulting in inhibition of its kinase activity. Accordingly, activated AKT2 blocked signaling down-stream of ASK1, including activation of JNK and p38 and the conversion of Bax to its active conformation. Expression of nonphosphorylatable ASK1-S83A overrode the AKT2-inhibited JNK/p38 activity and Bax conformational changes, whereas phosphomimic ASK1-S83D inhibited the effects of cisplatin on JNK/p38 and Bax. Cisplatin-induced Bax conformation change was inhibited by inhibitors or dominant negative forms of JNK and p38. In conclusion, our data indicate that AKT2 inhibits cisplatin-induced JNK/p38 and Bax activation through phosphorylation of ASK1 and thus, plays an important role in chemoresistance. Further, regulation of the ASK1/JNK/p38/Bax pathway by AKT2 provides a new mechanism contributing to its antiapoptotic effects. This work has been published in *J. Biol. Chem.* 278:23432-23440, 2003 (see appendix).

3. FTI sensitizes ovarian cancer cells to apoptosis.

To determine if inhibition of AKT2 pathway sensitizes chemotherapeutic drug-induced programmed cell death in human ovarian cancer cells, cisplatin resistance A2780CP and C13 cells were treated with cisplatin together with or without PI3K inhibitor LY294002 and farnesyltransferase inhibitor (FTI), which has been shown by us to specifically inhibit PI3K/AKT2 pathway (4). Apoptosis was significantly induced by treating the cells with cisplatin/LY294002 or cisplatin/FTI as compared to cisplatin alone. These data indicate that PI3K/Akt pathway is a critical target for ovarian cancer intervention. The reagents targeting PI3K or Akt could be potential drugs for ovarian cancer treatment, especially in chemoresistant tumors.

4. GGTI inhibits AKT2 and surviving pathways to induced apoptosis in ovarian cancer cells regardless p53 status.

Geranylgeranyltransferase I inhibitors (GGTIs) represent a new class of anti-cancer drugs. However, the mechanism by which GGTIs inhibit tumor cell growth is still unclear. Here, we demonstrate that GGTI-298 induces apoptosis in both cisplatin sensitive and resistant human ovarian epithelial cancer cells by inhibition of PI3K/AKT1/2 and survivin pathways. Following GGTI-298 treatment, kinase levels of PI3K and AKT1/2 were decreased and survivin expression was significantly reduced. Ectopic expression of either constitutively active AKT2 or survivin rescues human cancer cells from GGTI-298-induced apoptosis. Previous studies have shown that Akt mediates growth factor-induced survivin, whereas p53 inhibits survivin expression.

However, constitutively active AKT2 failed to rescue GGTI-298 downregulation of survivin. Further, GGTI-298 suppresses survivin expression and induces programmed cell death in both wild type p53 and p53-deficient ovarian cancer cell lines. These data indicate that GGTI-298 induces apoptosis by targeting PI3K/AKT and survivin parallel pathways independent of p53, and thus, GGTI-298 could be a valuable agent to overcome anticancer drug resistance (see appendix).

Key Research Accomplishment

1 Activation of AKT2 in human ovarian cancer contributes to chemoresistance.
2 AKT2 inhibits of cisplatin-induced JNK/p38 and Bax activation by phosphorylation of ASK1, suggesting AKT2 contributes to the development of drug resistance in ovarian cancer. Therefore, development reagent(s) to target AKT2 will greatly benefit to breast cancer intervention.
3 Inhibition of PI3K/AKT2 pathway by PI3K inhibitor and FTI sensitizes cisplatin resistant ovarian cancer cells to apoptosis.
4 GGTI overcomes cisplatin resistance in ovarian cancer cells with either mutant or wild type p53.

Reportable Outcomes

Publication:

1 Yuan Z, Feldman RI, Sussman GE, Coppola D, Nicosia SV, Cheng JQ. AKT2 Inhibition of Cisplatin-induced JNK/p38 and Bax Activation by Phosphorylation of ASK1: IMPLICATION OF AKT2 IN CHEMORESISTANCE. *J. Biol. Chem.* 278: 23432-23440, 2003.

2 Dan HC, Coppola D, Jinag K, Liu A, Hamilton AD, Nicosia SV, Sebti SM, Cheng JQ. Inhibition of Phosphatidylinositol-3-OH Kinase/Akt and Survivin Pathways by Geranylgeranyltransferase I Inhibitor-298 Induces Apoptosis in Human Ovarian Cancer Cells. *Oncogene, Accepted*

3 Gritsko TM, Coppola D, Paciga JE, Yang L, Sun M, Shelley SA, Fiorica JV, Nicosia SV, Cheng JQ. Activation and Overexpression of Centrosome Kinase BTAK/Aurora-A in Human Ovarian Cancer. *Clin Cancer Res.* 9:1420-1426, 2003.

Abstract/presentation

1. Yuan Z, Feldman RI, Sussman GE, Coppola D, Nicosia SV, Cheng JQ. AKT2 Inhibition of Cisplatin-induced JNK/p38 and Bax Activation by Phosphorylation of ASK1: Implication of AKT2 in Chemoresistance. 94th American Association for Cancer Research Annual Meeting, 2203.

2. Gritsko TM, Coppola D, Paciga JE, Yang L, Sun M, Shelley SA, Fiorica JV, Nicosia SV, Cheng JQ. Alterations of Aurora-A oncogene in human ovarian cancer. 94th American Association for Cancer Research Annual Meeting, 2203.
3. Yuan Z, Kaneko S, Coppola D, Nicosia SV, Cheng JQ. Akt-interacting protein, APαB, mediates PI3K/Akt survival signal through activation of PAK1 and NFκB pathways. 94th American Association for Cancer Research Annual Meeting, 2203.
4. Kaneko S, Yang L, Paciga J, Yu H, Nicosia SV, Jove R, Cheng JQ. Transcriptional Upregulation of AKT2 by Stat3 and Src. 19th Annual Meeting on Oncogene, 2003.
5. Dan DC, Sun M, Feldman RI, Nicosia SV, Wang H-G, Tsang B, Cheng, JQ. Akt Regulates X-linked inhibitor of apoptosis, XIAP: A Mechanism to Antagonize Cisplatin-Induced Apoptosis in Human Ovarian Epithelial Cancer Cells. 19th Annual Meeting on Oncogene, 2003.
6. Sun M, Yuan Z, Yang L, Feldman RI, Yeatman TJ, Jove R, Nicosia SV, Cheng JQ. AKT2 Inducible Gene hHb1-ΔN Feedback Activates PI3K/Akt Pathway and Promotes Cell Proliferation and Cell Survival. 19th Annual Meeting on Oncogene, 2003.
7. Yang L, Sun M, Yang H, Dan HC, Nicosia SV, Cheng JQ. Akt/PKB Inhibits HtrA2/Omi-stimulated Apoptosis through Direct Disruption of its Serine Protease Activity. 19th Annual Meeting on Oncogene, 2003.
8. Yuan Z, Feldman RI, Sussman GE, Coppola D, Nicosia SV, Cheng JQ. ASK1 is a target to overcome Akt induced cisplatin resistance. 19th Annual Meeting on Oncogene, 2003.
9. Cheng JQ. Akt and prenylation inhibitors as potential reagents to overcome p53-assocated chemoresistance in human ovarian cancer. The Gynecologic Oncology Group Meeting, 2003.

Conclusions

1. AKT2 pathway plays a pivotal role in ovarian cancer chemoresistance.
2. AKT2 inhibition of JNK/p38 and Bax by phosphorylation of ASK1.
3. FTI and GGTI overcome cisplatin resistance in human ovarian cancer.

References

2. Sun M, Wang, G, Paciga, JE, Feldman RI, Yuan Z, Ma X, Shelley SA, Jove R, Tsichlis PN, Nicosia SV, Cheng J.Q. AKT1/PKBα kinase is frequently elevated in human cancers and its constitutive activation is required for oncogenic transformation in NIH3T3 cells. *Am. J. Pathology* 159:431-437, 2001.

3. Yuan, Z., Sun, M., Feldman, R.I., Wang, G., Ma, X., Coppola, D., Nicosia, S.V. and Cheng, J.Q. Frequent activation of AKT and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/Akt pathway in human ovarian cancer. *Oncogene* 19:2324-2330, 2000.

4. .Cheng J.Q., Jiang X., Fraser M., Li M., Dan H.C., Sun M., Tsang B.K. Role of X-linked Inhibitor of Apoptosis Protein in chemoresistance in Ovarian Cancer: Possible involvement of the PI 3-Kinase/Akt Pathway. Drug Resist Updat. 5:131-146, 2002.

5. Jiang, K., Coppola, D., Crespo, N.C., Nicosia, S.V., Hamilton, A.D., Sebti, S.M. and Cheng, J.Q. The phosphoinositide 3-OH kinase/AKT2 pathway as a critical target for farnesyltransferase inhibitor-induced apoptosis. *Mol. Cell. Biol.,* 20:139-148, 2000.

Appendices

1 Yuan Z, Feldman RI, Sussman GE, Coppola D, Nicosia SV, Cheng JQ. AKT2 Inhibition of Cisplatin-induced JNK/p38 and Bax Activation by Phosphorylation of ASK1: IMPLICATION OF AKT2 IN CHEMORESISTANCE. *J. Biol. Chem.* 278: 23432-23440, 2003.

2 Dan HC, Coppola D, Jinag K, Liu A, Hamilton AD, Nicosia SV, Sebti SM, Cheng JQ. Inhibition of Phosphatidylinositol-3-OH Kinase/Akt and Survivin Pathways by Geranylgeranyltransferase I Inhibitor-298 Induces Apoptosis in Human Ovarian Cancer Cells. *Oncogene, Accepted*

3 Gritsko TM, Coppola D, Paciga JE, Yang L, Sun M, Shelley SA, Fiorica JV, Nicosia SV, Cheng JQ. Activation and Overexpression of Centrosome Kinase BTAK/Aurora-A in Human Ovarian Cancer. *Clin Cancer Res.* 9:1420-1426, 2003.

- Curriculum Vitae

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 26, Issue of June 27, pp. 23432–23440, 2003
Printed in U.S.A.

AKT2 Inhibition of Cisplatin-induced JNK/p38 and Bax Activation by Phosphorylation of ASK1

IMPLICATION OF AKT2 IN CHEMORESISTANCE*

Received for publication, March 17, 2003, and in revised form, April 11, 2003
Published, JBC Papers in Press, April 15, 2003, DOI 10.1074/jbc.M302674200

Zeng-qiang Yuan‡§¶, Richard I. Feldman‖, Gene E. Sussman‡, Domenico Coppola‡, Santo V. Nicosia‡, and Jin Q. Cheng‡§**

From the ‡Department of Pathology and Molecular Oncology and §Drug Discovery Program, University of South Florida College of Medicine and H. Lee Moffitt Cancer Center, Tampa, Florida 33612 and ‖Department of Cancer Research, Berlex Biosciences, Richmond, California 94804

Cisplatin and its analogues have been widely used for treatment of human cancer. However, most patients eventually develop resistance to treatment through a mechanism that remains obscure. Previously, we found that AKT2 is frequently overexpressed and/or activated in human ovarian and breast cancers. Here we demonstrate that constitutively active AKT2 renders cisplatin-sensitive A2780S ovarian cancer cells resistant to cisplatin, whereas phosphatidylinositol 3-kinase inhibitor or dominant negative AKT2 sensitizes A2780S and cisplatin-resistant A2780CP cells to cisplatin-induced apoptosis through regulation of the ASK1/JNK/p38 pathway. AKT2 interacts with and phosphorylates ASK1 at Ser-83 resulting in inhibition of its kinase activity. Accordingly, activated AKT2 blocked signaling downstream of ASK1, including activation of JNK and p38 and the conversion of Bax to its active conformation. Expression of nonphosphorylatable ASK1-S83A overrode the AKT2-inhibited JNK/p38 activity and Bax conformational changes, whereas phosphomimic ASK1-S83D inhibited the effects of cisplatin on JNK/p38 and Bax. Cisplatin-induced Bax conformation change was inhibited by inhibitors or dominant negative forms of JNK and p38. In conclusion, our data indicate that AKT2 inhibits cisplatin-induced JNK/p38 and Bax activation through phosphorylation of ASK1 and thus, plays an important role in chemoresistance. Further, regulation of the ASK1/JNK/p38/Bax pathway by AKT2 provides a new mechanism contributing to its antiapoptotic effects.

Although cisplatin and its analogues, the DNA cross-linking agents, are first-line chemotherapeutic agents for the treatment of human ovarian and breast cancers, chemoresistance remains a major hurdle to successful therapy (1, 2). Several molecules have been implicated in cisplatin resistance, including decreased cellular detoxication (3, 4), increased DNA repair (5), and mutations of *p53* tumor suppressor gene (6, 7). However, the mechanisms involved in cisplatin resistance are still poorly understood. A growing body of evidence indicates that defects in the intra- and extracellular survival/apoptotic pathways are an important cause of resistance to cytotoxic agents.

Phosphatidylinositol 3-kinase (PI3K)[1]/Akt is a major cell survival pathway that has been extensively studied recently (8). PI3K is a heterodimer composed of a p85 regulatory and a p110 catalytic subunit and converts the plasma membrane lipid phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate. Pleckstrin homology domain-containing proteins, including Akt, accumulate at sites of PI3K activation by directly binding to phosphatidylinositol 3,4,-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate. Akt (also known as PKB) represents a subfamily of serine/threonine kinases. Three member of this family, including AKT1, AKT2, and AKT3, have been identified so far. Akt is activated in a PI3K-dependent manner by a variety of stimuli, including growth factors, protein phosphatase inhibitors, and stress (9–12). Downstream targets of Akt contain the consensus phosphorylation sequence RXRXX(S/T)(F/L) (13). Several targets of Akt that have been identified have roles in the regulation of apoptosis, such as the proapoptotic proteins BAD and caspase-9 and transcription factor FKHRL1. Phosphorylation by Akt blocks BAD binding to Bcl-x_L, inhibits caspase-9 protease activity, and blocks FKHRL1 function, reducing Fas ligand transcription (14–16).

Among Akt family members, AKT2 has been shown to be predominantly involved in human malignancies including ovarian cancer. We have demonstrated previously amplification of the AKT2 in a number of human ovarian cancer cell lines and recently detected frequently elevated protein and kinase levels of AKT2 in about a half of primary ovarian carcinoma examined (17, 18). Moreover, ectopic expression of wild type of AKT2 but not Akt1 in NIH 3T3 cells resulted in malignant transformation (19). Inhibition of PI3K/AKT2 by farnesyltransferase inhibitor-277 induced apoptosis in ovarian cancer cells that overexpress AKT2 (20). We have also shown that TNFα and extracellular stresses, including UV irradiation, heat shock, and hyperosmolarity, induce AKT2 kinase and that

* This work was supported in part by NCI, National Institutes of Health Grants CA77935 and CA89242 and United States Department of Defense Grants DAMD 17-01-1-0397 (to Z.-Q. Y.), DAMD17-00-0559, DAMD 17-01-1-0394, and DAMD17-02-1-0671. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Predoctoral Fellowship awardee from the United States Department of Defense.

** To whom correspondence should be addressed: Dept. of Pathology, University of South Florida College of Medicine and H. Lee Moffitt Cancer Center, 12901 Bruce B. Downs Blvd., MDC Box 11, Tampa, FL 33612. Tel.: 813-974-8595; Fax: 813-974-5536; E-mail: jcheng@hsc.usf.edu.

[1] The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; ASK1, apoptosis signal-regulating kinase 1; JNK, c-Jun NH_2-terminal kinase; PARP, poly(ADP-ribose) polymerase; HA, hemagglutinin; DMEM, Dulbecco's modified Eagle's medium; TNFα, tumor necrosis factor α; GST, glutathione *S*-transferase; HEK, human embryonic kidney; MKK, mitogen-activated protein kinase kinase.

activated AKT2 inhibits JNK/p38 activity to protect cells from TNFα and cellular stress-induced apoptosis (21).

JNK and p38 are predominantly activated through environmental stresses, including osmotic shock, UV radiation, heat shock, oxidative stress, protein synthesis inhibitors, stimulation of Fas, and inflammatory cytokines such as TNFα and interleukin-1. Stimulation of JNK/p38 activity has also been shown to be critical for cisplatin-induced apoptosis in some cancer cells (22, 23). Specific inhibition of JNK or p38, through small molecule inhibitors, dominant negative JNK/p38 mutants, or knock-out of JNK expression, suppresses various types of stress-induced apoptosis (24). Although it has been shown that JNK phosphorylates and inhibits antiapoptotic protein Bcl-2 (25), the mechanism of JNK/p38 induction of apoptosis is still not well understood.

Apoptosis signal-regulating kinase 1 (ASK1) is a member of the mitogen-activated protein kinase kinase kinase family that activates both the SEK1-JNK and MKK3/MKK6-p38 signaling cascades (26–28). ASK1 is a general mediator of cell death in responds to a variety of stimuli, including oxidative stress (29, 30) and chemotherapeutic drugs such as cisplatin and paclitaxel (22, 23). Ectopic expression of ASK1 induced apoptosis in various cell types (26, 28). Furthermore, disruption of the *ASK1* gene in mice causes a remarkable reduction in sensitivity to stress-induced cell death, such as that promoted by TNFα or oxidative stress (33). These data indicate that ASK1 plays a key proapoptotic function through promoting the sustained activation of JNK/p38 mitogen-activated protein kinases.

In the present study, we show that AKT2 activity promotes resistance to cisplatin-induced apoptosis in A2780S ovarian cancer cells through the inhibition of the ASK1/JNK/p38 pathway. In A2780S cells, we show that AKT2 complexes with and phosphorylates ASK1 at Ser-83 within a consensus Akt phosphorylation site on this molecule. This results in inhibition of ASK1 activity and the blocking of JNK and p38 activation. We also show that these latter activities are required for cisplatin-induced apoptosis in A2780S cells. Furthermore, in response to cisplatin, we observe that ASK1 and JNK/p38 promote Bax conformational change. Collectively, these studies indicate that AKT2 may be an important mediator of chemoresistance through its regulatory effects on the ASK1/JNK/p38/Bax pathway.

EXPERIMENTAL PROCEDURES

Reagents—Cisplatin, LY294002, and anti-Bax (6A7) were obtained from Sigma. DMEM and fetal bovine serum were purchased from Invitrogen. Anti-phospho-Akt (Ser-473), anti-cleaved PARP, anti-phospho-JNK (p54/44), anti-phospho-extracellular signal-regulated kinase 1/2(44/42), anti-phospho-p38, anti-phospho-mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1/2, and anti-mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1/2 antibodies were obtained from Cell Signaling (Beverly, MA). GST-c-Jun and GST-ATF6 were also purchased from Cell Signaling. Anti-AKT2, anti-Bax, and anti-ASK1 were obtained from Santa Cruz Biotechnology. JNK inhibitor II and p38 inhibitor SB203580 were from Calbiochem.

Cell Culture and Cisplatin Treatment—The human epithelial cancer cell lines, A2780S and A2780CP, kindly provided by Benjamin K. Tsang at The Ottawa Hospital, and human embryonic kidney (HEK) 293 cells were cultured at 37 °C and 5% CO_2 in DMEM supplemented with 10% fetal bovine serum. The cells were seeded in 60-mm Petri dishes at a density of 0.5×10^6 cells per dish. After 24 h, cells were treated with cisplatin (20 μM) for the appropriate time as noted in the figure legends.

Expression Constructs—The cytomegalovirus-based expression constructs encoding wild type HA-AKT2 and constitutively active HA-Myr-AKT2 have been described previously (31). The pcDNA$_3$-HA-ASK1 construct was kindly provided by Hidenori Ihijo at Tokyo Medical and Dental University. HA-ASK1-S83A and ASK1-S83D, as well as dominant negative AKT2 with triple mutations (T309A, E299K, and S474A), were created using the QuikChange site-directed mutagenesis kit (Stratagene). JNK and p38 plasmids were obtained from Roger Davis at the University of Massachusetts.

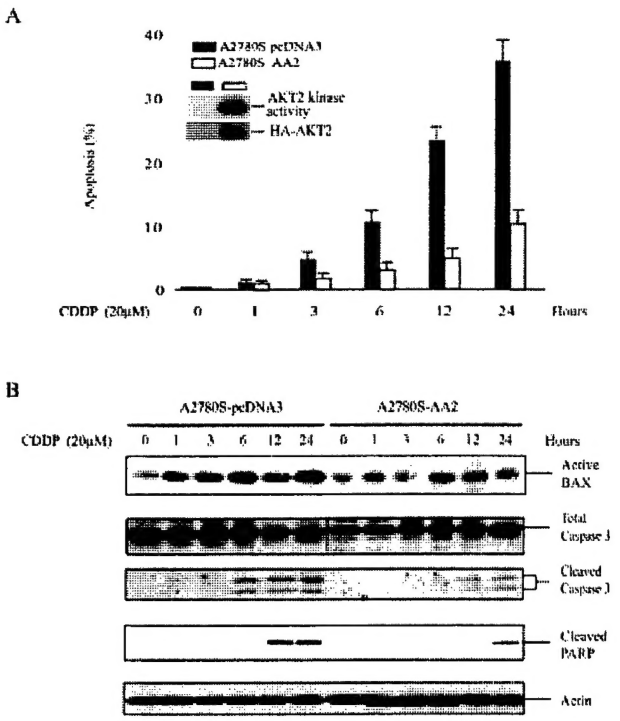


FIG. 1. **Activation of AKT2 renders cells resistant to cisplatin and inhibits cisplatin-induced Bax conformational change and caspase-3 cleavage.** *A*, cisplatin-sensitive A2780S cells were stably transfected with constitutively active AKT2 (*A2780S-AA2*) or pcDNA3 (*A2780S-pcDNA3*). Expression and kinase activity of transfected AKT2 were examined by Western blot and *in vitro* kinase assays (*inset*). The cells were treated with cisplatin (*CDDP*; 20 μM) for indicated time and analyzed by Tunel assay. Apoptotic cells were quantified in triple experiments. *B*, Western blot analysis. The cells were treated with cisplatin and lysed. A portion of lysate was immunoprecipitated with anti-active Bax (6A7) and detected with anti-total Bax antibody (*top panel*). The rest of the lysates were immunoblotted and probed with anti-caspase-3 (*second and third panels*), anti-PARP (*fourth panel*), and anti-actin (*bottom panel*) antibodies.

Immunoprecipitation and Immunoblotting—Cells were lysed in a buffer containing 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 15% (v/v) glycerol, 1% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin and leupeptin, 2 mM benzamidine, 20 mM NaF, 10 mM NaPPi, 1 mM sodium vanadate, and 25 mM β-glycerolphosphate. Lysates were centrifuged at 12,000 $\times$ *g* for 15 min at 4 °C prior to immunoprecipitation or Western blot. Aliquots of the cell lysates were analyzed for protein expression and enzyme activity. For immunoprecipitation, lysates were precleared with protein A-protein G (2:1)-agarose beads at 4 °C for 20 min. Following the removal of the beads by centrifugation, lysates were incubated with appropriate antibodies in the presence of 25 μl of protein A-protein G (2:1)-agarose beads for at least 2 h at 4 °C. The beads were washed with buffer containing 50 mM Tris-HCl (pH 7.5), 0.5 M LiCl, and 0.5% Triton X-10; twice with phosphate-buffered saline; and once with buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 1 mM dithiothreitol, all supplemented with 20 mM β-glycerolphosphate and 0.1 mM sodium vanadate. The immunoprecipitates were subjected to *in vitro* kinase assay or Western blotting analysis. Protein expression was determined by probing Western blots of immunoprecipitates or total cell lysates with the appropriate antibodies as noted in the figure legends. Detection of antigen-bound antibody was carried out with the ECL Western blotting analysis system (Amersham Biosciences).

In Vitro Kinase Assay—Protein kinase assays were performed as described previously (21). Briefly, reactions were carried out in the presence of 10 μCi of [γ-^{32}P]ATP (PerkinElmer Life Sciences) and 3 μM cold ATP in 30 μl of buffer containing 20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$, and 1 mM dithiothreitol. 2 μg of myelin basic protein was used as the exogenous substrate. After incubation at room temperature for 30 min the reaction was stopped by adding protein loading buffer, and proteins were separated on SDS-PAGE gels. Each

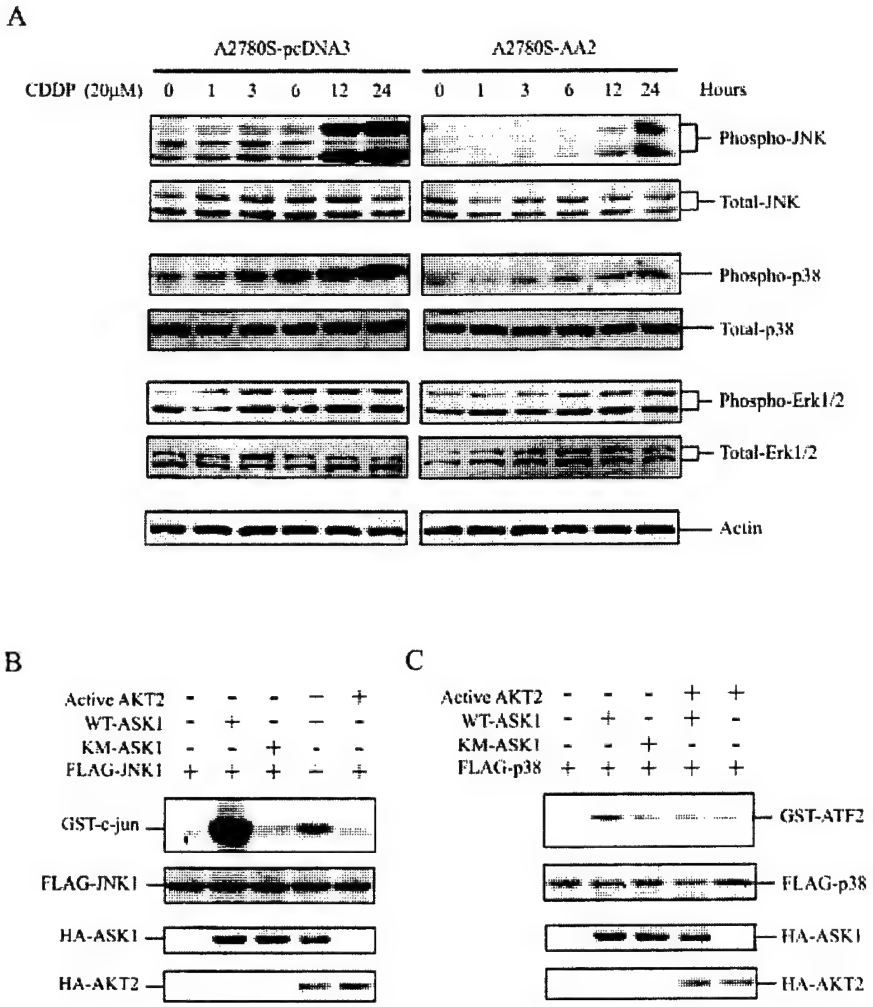


FIG. 2. **AKT2 inhibits JNK and p38 activation induced by cisplatin and ASK1.** *A*, immunoblotting analysis. Following treatment with cisplatin at indicated times, the cells were lysed and immunoblotted. The blots were detected with indicated antibodies. *B* and *C*, *in vitro* kinase assay. HEK293 cells were transfected with the indicated expression plasmids. After 36 h of transfection, cells were lysed and immunoprecipitated with anti-FLAG antibody. The FLAG-JNK and FLAG-p38 immunoprecipitates were subjected to *in vitro* kinase using GST-c-Jun (*B*) and GST-ATF2 (*C*) as substrate, respectively (*top panel*). Expression of the transfected plasmids was shown in the *second*, *third*, and *fourth panels*.

experiment was repeated three times, and the relative amounts of incorporated radioactivity were determined by autoradiography and quantified with a PhosphorImager (Molecular Dynamics).

In Vivo [^{32}P]P$_i$ Labeling—HEK293 cells were co-transfected with active AKT2 and HA-tagged ASK1 or pcDNA3 and labeled with [^{32}P]P$_i$ (0.5 mCi/ml) in phosphate- and serum-free DMEM medium for 4 h. Cell lysates were subjected to immunoprecipitation with anti-HA antibody. The immunoprecipitates were separated by 7.5% SDS-PAGE and transferred to membranes. Phosphorylated ASK1 band was visualized by autoradiography. The expression of transfected ASK1 was detected with anti-HA antibody.

Luciferase Reporter Assay—Cells were seeded in 6-well plate and transfected with c-Jun or ATF6 reporter plasmid (pGl-GAL4), pSV2-β-gal, and different forms (wild type, constitutively active, or dominant negative) of HA-AKT2 together with or without different forms of ASK1 or vector alone. After 36 h of the transfection, luciferase and β-galactosidase assays were performed according to the manufacturer's procedures (Promega and Tropix, respectively). Each experiment was repeated three times.

Tunel Assay—Cells were seeded into 60-mm dishes and grown in DMEM supplemented with 10% fetal bovine serum for 24 h and treated with 20 μM cisplatin for different times. Apoptosis was determined by Tunel assay using an *in situ* cell death detection kit (Roche Applied Science). These experiments were performed in triplicate.

RESULTS

Activation of AKT2 Renders Cisplatin-sensitive Cells Resistant to Cisplatin and Inhibits Cisplatin-induced Bax Conformational Change—We have shown previously (18, 34) frequent activation of AKT2 kinase in human ovarian and breast cancers. To examine whether activation of AKT2 contributes to chemoresistance in cancer cells, cisplatin-sensitive A2780S cells were stably transfected with constitutively active AKT2 (A2780S-AA2) or pcDNA3 vector alone. Expression and kinase activity of transfected constitutively active AKT2 were confirmed by Western blot and *in vitro* kinase analysis (Fig. 1*A*, *inset*). Following treatment with cisplatin (20 μM) for 0, 1, 3, 6, 12, and 24 h, programmed cell death in A2780S-pcDNA3 and A2780S-AA2 (active AKT2) cells were examined by Tunel assay. The number of apoptotic cells was quantified by counting three different microscopic fields. Three h after treatment, A2780S-pcDNA3 cells begun to undergo apoptosis. By 24 h of treatment, 35% of the cells were apoptotic, which is a similar response reported in the literature for parental A2780S cells (35). However, we observed a distinctly lower percentage of apoptotic cells at the time points 3, 6, 12, and 24 h in A2780S-AA2 cells (Fig. 1*A*), indicating that activation of AKT2 renders cisplatin-sensitive A2780S cells resistant to cisplatin.

It has been shown that Bax is required for cisplatin-induced apoptosis, *i.e.* cisplatin activates Bax by inducing its N-terminal conformation change and then targeting it to mitochondria resulting in cytochrome *c* release and activation of apoptotic pathway (36, 37). Thus, we next examined the effects of AKT2 activation on induction of Bax conformational changes by cisplatin. After treatment with cisplatin, A2780S-pcDNA3 and A2780S-AA2 cells were lysed and immunoprecipitated with anti-active Bax (6A7) antibody. The immunoprecipitates were subjected to Western blot analysis with total anti-Bax anti-

body. As shown in Fig. 1*B*, cisplatin promotes alteration of Bax conformation after 3 h of treatment in A2780S-pcDNA3 cells but not in A2780S-AA2 cells. Accordingly, cleavage of caspase 3 and its substrate, PARP, was also inhibited by expression of constitutively active AKT2 as compared with pcDNA3-transfected A2780S cells (Fig. 1*B*).

AKT2 Inhibits Cisplatin- and ASK1-induced JNK and p38 Activation—It has been documented that stress kinases, JNK and p38, are activated by cisplatin, and their activations are required for cisplatin-induced programmed cell death (22, 23, 38). To examine whether the effect of cisplatin on JNK and p38 is abrogated by the activation of AKT2, A2780S-pcDNA3 and A2780S-AA2 cells were treated with cisplatin at different times. As expected, JNK and p38 were activated by cisplatin in A2780S-pcDNA3 cells, and the activation of p38 took place before that of JNK. However, the activation of JNK and p38 was reduced dramatically in A2780S cells transfected with a constitutively active AKT2. No significant difference in the phosphorylation levels of extracellular signal-regulated kinase was observed between these two cell lines (Fig. 2*A*).

To explore the mechanism of AKT2 inhibition of the JNK and p38, we probed for direct interaction of these proteins by coimmunoprecipitation. We were not, however, able to demonstrate any interaction between AKT2 and JNK or p38 (data not shown). As ASK1 is known to activate JNK/p38 and be induced by cisplatin (32), and its overexpression is sufficient to induce apoptosis (26, 28), we next examined whether AKT2 restrains JNK and p38 activity through inhibition of ASK1. HEK293 cells were transfected with FLAG-JNK1 or FLAG-p38 and wild type or kinase-dead ASK1 (KM-ASK1), with or without constitutively active AKT2. After 36 h of transfection, cells were lysed and immunoprecipitated with anti-FLAG antibody. FLAG-JNK1 and FLAG-p38 immunoprecipitates were subjected to *in vitro* kinase assays using GST-c-Jun and GST-ATF2 as substrates, respectively. Repeated experiments revealed that kinase activities of JNK1 and p38 were significantly induced by expression of wild type but not kinase-dead ASK1 and that the activation of JNK and p38 was attenuated by ectopic expression of constitutively active AKT2 (Fig. 2, *B* and *C*). These data indicate that AKT2 may negatively regulate ASK1, causing inhibition of cisplatin-induced JNK/p38 activation and apoptosis.

AKT2 Interacts with, Phosphorylates, and Inhibits ASK1—To examine whether ASK1 is a direct target of AKT2, co-immunoprecipitation was carried out with anti-AKT2 antibody and detected with anti-ASK1 antibody, and vice versa. As shown in Fig. 3, *A* and *B*, interaction between ASK1 and AKT2 was readily detected, and this interaction was enhanced by cisplatin treatment. Sequence analysis revealed that an AKT2 phosphorylation consensus site resides in ASK1 at residue Ser-83, which is conserved between human and mouse. To determine whether AKT2 phosphorylates ASK1, *in vitro* AKT2 kinase assays were performed using immunoprecipitated HA-ASK1 (wild type ASK1 or ASK1S83A) as substrates (Fig. 3*C*). In addition, *in vivo* [^{32}P] labeling and immunoblotting analyses with anti-phospho-Ser/Thr Akt substrate antibody were carried out in HEK293 cells transfected with ASK1 and constitutively active or wild type AKT2 (Fig. 3*D*). Both *in vitro* kinase and *in vivo* labeling experiments, as well as Western blot analysis, showed that wild type and constitutively active AKT2 phosphorylate ASK1 at Ser-83 with the lower phosphorylation level by wild type AKT2 (Fig. 3, *C* and *D*).

We next determined whether cisplatin-induced ASK1 activation is inhibited by AKT2 and, if it is, whether this inhibition depends upon AKT2 phosphorylation of ASK1 at Ser-83. Mutagenesis was used to create a form of ASK1 not phosphorylatable by AKT2, ASK1-S83A, prepared by converting Ser-83 of ASK1 to alanine. We also prepared ASK1-S83D, derived from mutating Ser-83 of ASK1 to aspartic acid, which mimics ASK1 phosphorylated by AKT2. A2780S cells were transfected with ASK1-S83A or ASK1-S83D, with or without constitutively active AKT2. Following cisplatin treatment, ASK1s were immunoprecipitated, and *in vitro* ASK1 kinase assays were performed using myelin basic protein as substrate. As shown in Fig. 3*E*, cisplatin significantly induced the kinase activity of both wild type ASK1 and nonphosphorylatable ASK1-S83A but not AKT2 phosphomimic ASK1-S83D. Expression of constitutively active AKT2 inhibited cisplatin-stimulated kinase activity of wild type ASK1 but not that of nonphosphorylatable ASK1-S83A. These results indicate that ASK1 kinase activity is negatively regulated by AKT2 through phosphorylation of ASK1 at Ser-83.

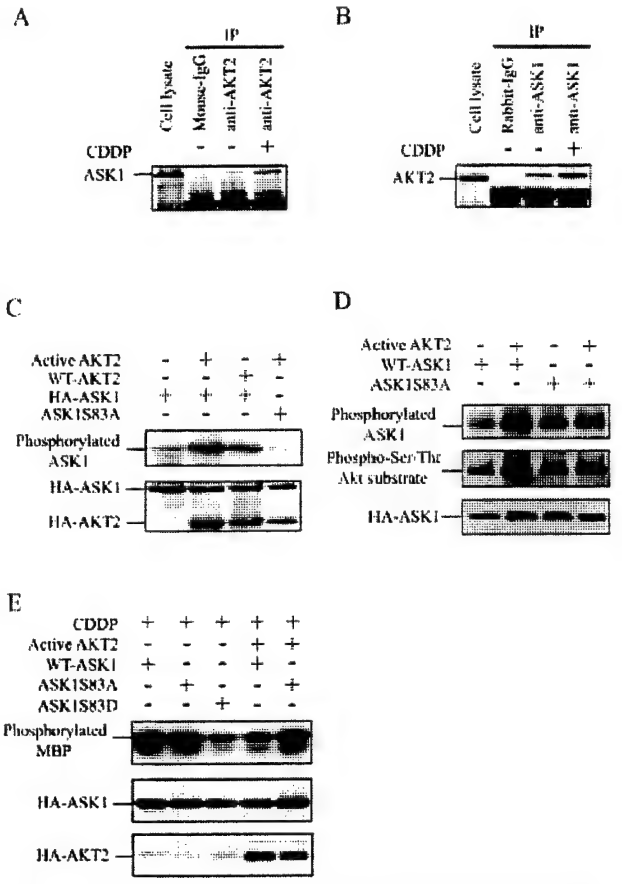


FIG. 3. **AKT2 interacts with and phosphorylates ASK1 and inhibits ASK1 kinase activity.** *A* and *B*, Western blot analyses of the immunoprecipitates prepared from A2780S cells treated with or without cisplatin. Immunoprecipitation was performed with anti-AKT2 and detected with anti-ASK1 antibody (*A*) and vice versa (*B*). *C*, *in vitro* kinase analysis of AKT2 immunoprecipitates derived from HEK293 cells that were transfected with indicated plasmids. Immunopurified HA-ASK1 or HA-ASK1-S83A was used as substrate (*top panel*). *The bottom panel* shows expression of transfected plasmids. *D*, *in vivo* [^{32}P]P_i labeling. HEK293 cells were transfected with indicated expression constructs, labeled with [^{32}P]P_i (0.5 mCi/ml), and immunoprecipitated with anti-HA antibody. The HA-ASK1 immunoprecipitates were separated in SDS-PAGE, blotted, and exposed to x-ray film (*top panel*). The membrane was then detected with anti-Akt substrate antibody (*middle panel*) and anti-HA antibody (*bottom panel*). *E*, *in vitro* ASK1 kinase analysis of the immunoprecipitates prepared from A2780S cells transfected with indicated plasmids and treated with cisplatin (20 μM) for 6 h. Myelin basic protein was used as substrate (*top panel*). Expression of transfected different forms of ASK1 and AKT2 was shown in the *second and third panels*.

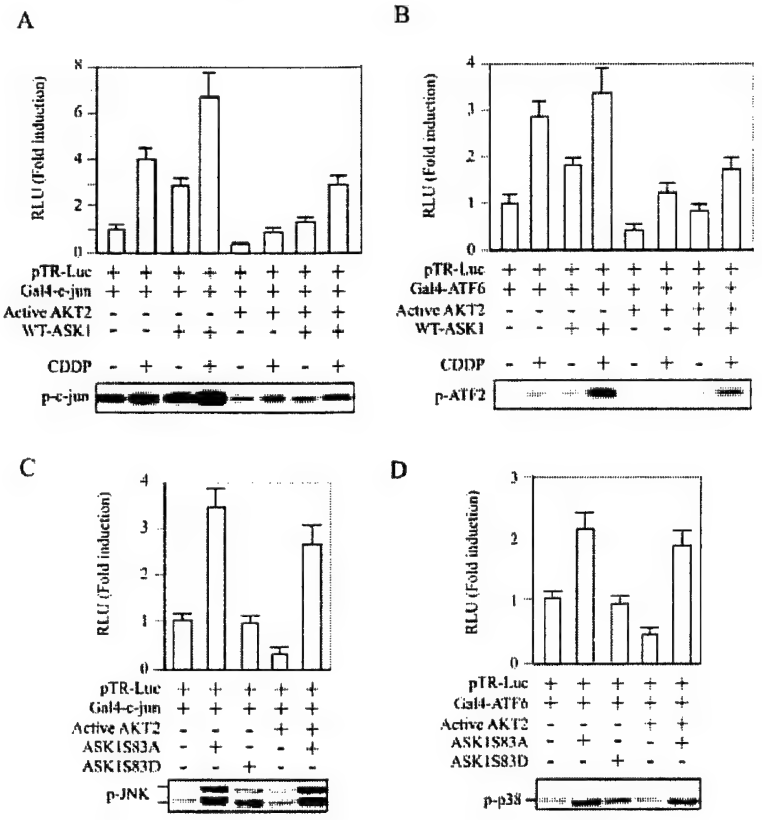


FIG. 4. **Activation of AKT2 inhibits ASK1- and/or cisplatin-induced JNK and p38 activation.** *A–D*, luciferase reporter assays. A2780S cells were transfected with indicated expression constructs and treated with or without cisplatin. Luciferase and β-galactosidase assays were performed, and the reporter activity was normalized by dividing luciferase activity with β-galactosidase. Each experiment was repeated three times. The *bottom panels* of *A* and *B* show the results obtained from *in vitro* JNK and p38 kinase assays using GST-c-Jun and ATF2 as substrates, respectively. The effects of AKT2 and its phosphorylation of ASK1 at Ser-83 on JNK and p38 activation were shown in the *bottom panels* of *C* and *D*.

AKT2 Inhibition of Cisplatin-stimulated JNK and p38 Is Mediated by Phosphorylation of ASK1 at Residue Ser-83—We next determined whether phosphorylation of ASK1 on Ser-83 by AKT2 is required for AKT2 inhibition of p38 and JNK, which are downstream from ASK1. Luciferase reporter assays were performed using Gal4-c-Jun/pTR-Luc (for JNK) and Gal4-ATF6/pTR-Luc (for p38) reporter systems. A2780S cells were transfected with ASK1, ASK1-S83A, ASK1-S83D, and/or Myr-AKT2, as well as pTR-Luc, Gal4-c-Jun, or Gal4-ATF6, and treated with or without cisplatin. Three independent experiments revealed that cisplatin induces Gal4-c-Jun or Gal4-ATF-regulated reporter activities. Further, *in vitro* JNK and p38 kinase analysis revealed that the phosphorylation of c-Jun and ATF2 was also stimulated by cisplatin treatment. These effects were enhanced by ectopic expression of wild type ASK1; however, they were inhibited by expression of constitutively active AKT2 (Fig. 4, *A* and *B*). Expression of nonphosphorylatable ASK1-S83A was also sufficient to induce the reporter activities and to attenuate the inhibitory action of constitutively active AKT2. In contrast, phosphomimic ASK1-S83D failed to stimulate the reporter activities (Fig. 4, *C* and *D*). Moreover, the effects of ASK1-S83A and ASK1-S83D on cisplatin-induced JNK and p38 activation were similar to their action on Gal4-c-Jun and Gal4-ATF6 reporters (Fig. 5*A*). Therefore, we conclude that AKT2 inhibits cisplatin-induced JNK and p38 via a phosphorylation of ASK1-dependent manner.

Cisplatin-induced Bax Conformational Change Is Regulated by AKT2 Phosphorylation of ASK1—Previous studies have shown that JNK is required for UV- and cisplatin-induced Bax conformational change (39). Our data demonstrate that ectopic expression of constitutively active AKT2 overrides cisplatin-induced ASK1/JNK/p38 activation and prevents formation of the active Bax conformation (see Figs. 1 and 2). To more directly probe the effect of AKT2 phosphorylation of ASK1 on Bax activation, we transfected A2780S cells with nonphosphorylatable and phosphomimic ASK1 and treated the cells with or without cisplatin. As revealed by immunoprecipitation and Western blot analyses, ectopic expression of nonphosphorylatable ASK1-S83A enhance cisplatin-dependent Bax conformational change, whereas ASK1-S83D, mimicking ASK1 phosphorylated by AKT2, inhibited cisplatin-induced Bax

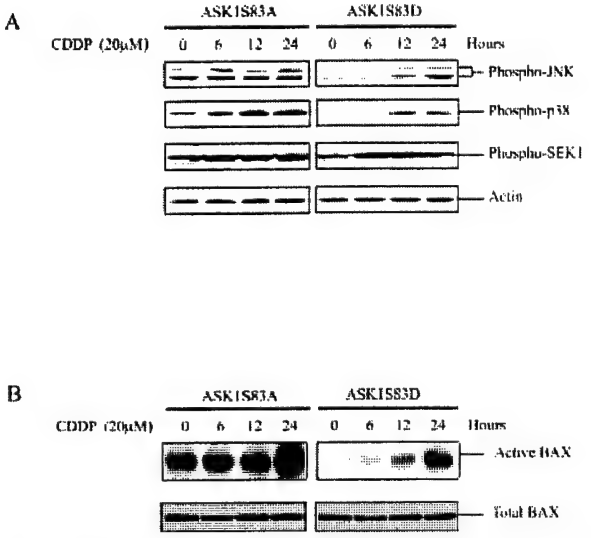


FIG. 5. **AKT2 phosphorylation of ASK1 at Ser-83 plays a critical role in cisplatin-induced JNK/p38 activation and Bax conformational change.** *A*, immunoblotting analysis of A2780S cells transfected with nonphosphorylatable and phosphomimic ASK1 prior to treatment with cisplatin. The blots were probed with the indicated antibodies. *B*, Western blot analysis. A2780S cells were transfected with indicated expression plasmids, treated with cisplatin, immunoprecipitated with anti-active Bax antibody, and detected with anti-total Bax antibody (*top panel*). Expression of Bax was shown in the *bottom panel*.

activation (Fig. 5*B versus* Fig. 1*B*). These data suggest that AKT2 inhibition of cisplatin-stimulated Bax conformational change is mediated at least to some extent by AKT2 phosphorylation of ASK1 at residue Ser-83.

Because JNK and p38 are downstream targets of ASK1, we next examined their roles in ASK1-stimulated Bax activation by using selective small molecule inhibitors of JNK and p38, JNK inhibitor II and SB 203580. As illustrated in Fig 6*A*, expression of ASK1 was sufficient to induce a Bax conformational change, and this effect was enhanced by cisplatin treatment. However, the conformational change of Bax induced by ASK1 and/or cisplatin was significantly diminished following treatment of cells with JNK inhibitor II (10 μM) and p38 inhibitor, SB 203580 (10 μM), suggesting that JNK and/or p38 mediate cisplatin-induced Bax activation. To probe the individual contributions of JNK and p38 in cisplatin-stimulated Bax activation, we further examined the effects of small molecule inhibitors of p38 and JNK and the expression of wild type and dominant negative forms of these kinases. A2780S cells were transfected with wild type or dominant negative JNK or p38, together with ASK1, and treated with or without cisplatin and/or inhibitor of JNK or p38. As shown in Fig. 6, *B* and *C*, expression of wild type JNK or p38 enhanced ASK1- and cisplatin-induced Bax activation, as expected. Furthermore, dominant negative JNK or a small molecule JNK inhibitor significantly decreased the Bax activation induced by cisplatin treatment or ectopic expression of ASK1 (Fig. 6*B*). We observed that only slight inhibition of the Bax activation was in the cells expressing dominant negative p38 or treated with p38 inhibitor (Fig. 6*C*). These results indicate that cisplatin- and/or ASK1-induced Bax activation is mediated primarily by JNK.

Inhibition of PI3K/AKT2 Pathway Sensitizes Cells to Cisplatin-induced Apoptosis—Because activated AKT2 reduces the cisplatin sensitivity of A2780S cells, we next examined the ability of inhibition of the PI3K/AKT2 pathway to sensitize cells to cisplatin-induced apoptosis. Cisplatin-resistant A2780CP and A2780S cells were transfected with dominant negative AKT2 or treated with PI3K inhibitor, LY294002, together with cisplatin. Tunel assay analyses revealed that either LY294002 or ectopic expression of dominant negative AKT2 enhanced cisplatin-induced apoptosis as compared with cells treated with cisplatin alone (Fig. 7, *A* and *C*). Accordingly, cleavage of caspase-3 and PARP was increased by treatment of cells with a combination of cisplatin with LY294002 or dominant negative-AKT2 (Fig. 7, *B* and *D*). To examine the role played by AKT2 phosphorylation of ASK1 in cisplatin-induced apoptosis, we transfected A2780S cells with ASK1-S83A, which is not phosphorylated by AKT2, ASK1-S83D, which mimics AKT2 phosphorylation, and then induced apoptosis with cisplatin. Notably, ectopic expression of ASK1-S83A significantly augmented cisplatin-induced apoptosis. In contrast, expression of ASK1-S83D conferred resistance to cisplatin (Fig. 7*E*). These data further indicate that PI3K/AKT2 promotes cell survival through phosphorylation and inhibition of ASK1 signaling.

DISCUSSION

We have demonstrated previously (18, 34) that AKT2 kinase is frequently elevated in human ovarian and breast cancers and that AKT2, like Akt1, exerts its anti-apoptotic function through phosphorylation of Bad (20). However, the biological role of AKT2 activation in human cancer and the mechanism of AKT2-induced cell survival in a chemotherapeutic setting have not been well documented. In this study, we show that activation of AKT2 significantly increases the resistance of ovarian cancer cells to cisplatin. AKT2 protects cells from cisplatin-induced apoptosis by inhibiting cisplatin-induced JNK/p38 activation and Bax conformational change.

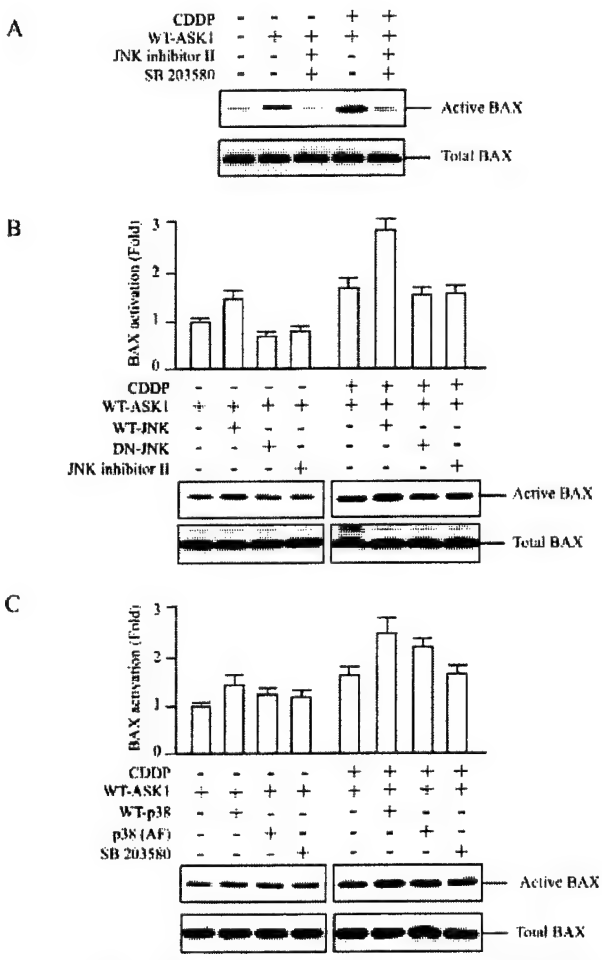


FIG. 6. **JNK and p38 mediate cisplatin- and ASK1-induced Bax conformational change.** *A*, Western blot analysis. A2780S cells were transfected with ASK1 and treated with JNK inhibitor II (10 μM) and SB 203580 (10 μM) for 1 h prior to addition of cisplatin. Following 16 h of the further treatment, Bax conformational change was examined as described above. *B* and *C*, immunoblotting analyses. A2780S cells were transfected with indicated plasmids and treated with indicated reagents. Bax conformational change was evaluated as described above. Both JNK inhibitor and dominant negative JNK exhibited more significant inhibitory effects on Bax activation than did p38 inhibitor and dominant negative p38 (AF). All the experiment was repeated three times.

AKT2 mediates these effects through its interaction and phosphorylation of ASK1.

Cisplatin-induced JNK and p38 activations are required for its anti-tumor activity (22, 23). This activation has been shown to correlate with induction of apoptosis by cisplatin (22, 23). Moreover, studies using dominant negative mutants of JNK and p38 and specific pharmacological inhibitors have shown that activation of JNK and/or p38 is necessary for stress or chemotherapeutic drug-induced apoptosis (38, 40). Also, studies on fibroblasts with targeted disruptions of all the functional *Jnk* genes established an essential role for JNK in UV- and other stress-induced apoptosis (41). ASK1, an upstream regulator of JNK/p38, has also been shown to be induced by cisplatin (32). Furthermore, oxidative stress-induced ASK1 kinase activity is inhibited by Akt1 (42). Consistent with this, we demonstrate that activation of AKT2 inhibits cisplatin-induced JNK and p38 through direct interaction with and phosphorylation of ASK1 at serine 83. We also demonstrate that phosphorylation of ASK1 by AKT2 renders cells resistant to cisplatin.

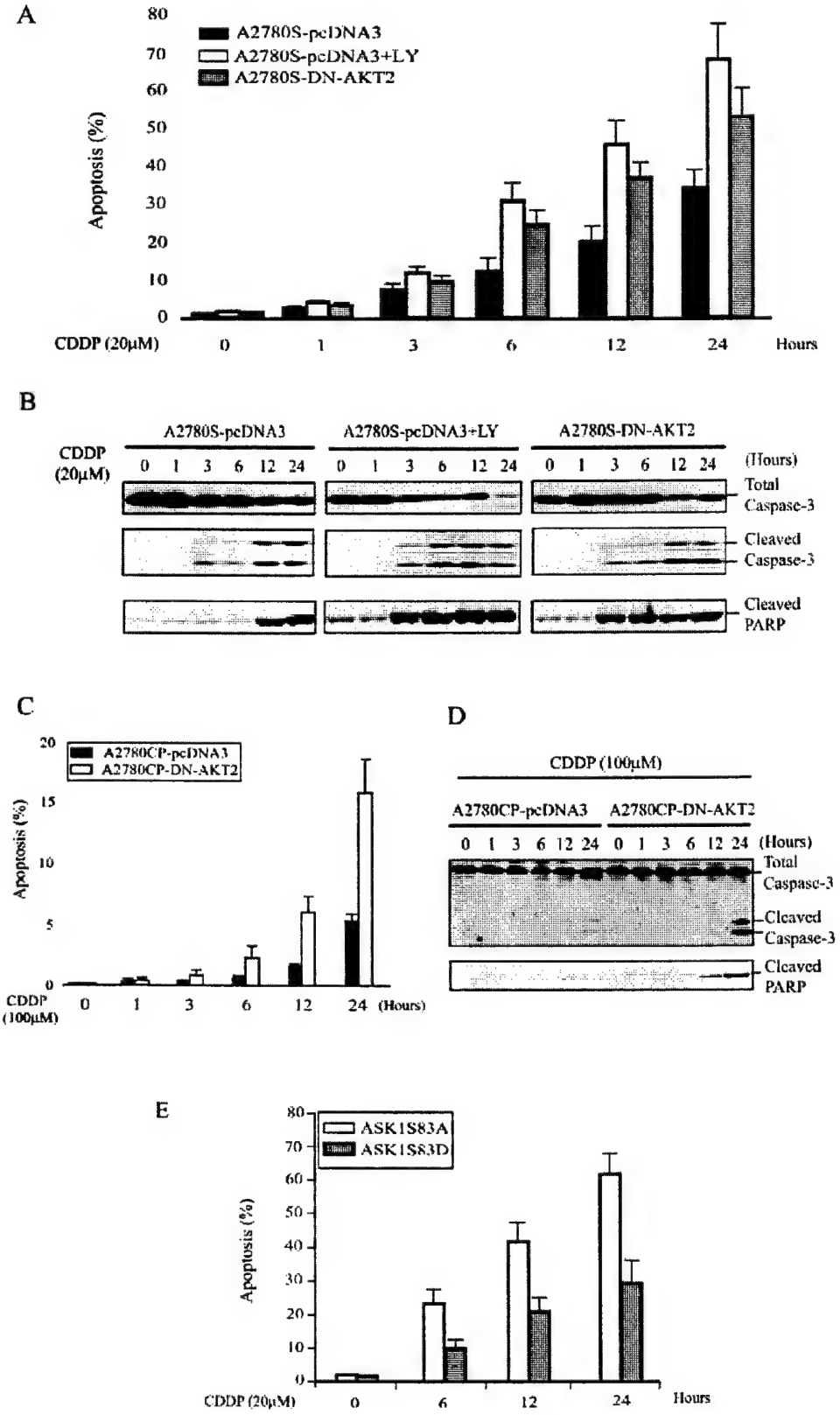


FIG. 7. **Inhibitions of PI3K/AKT2 and ASK1 phosphorylation sensitize cells to cisplatin-induced apoptosis.** *A*, Tunel assay. A2780S cells were transfected with dominant negative AKT2 or pcDNA3 vector and treated with cisplatin or cisplatin/LY294002. Apoptosis was examined and quantified after treatment for the indicated times. *B*, immunoblotting analysis of cell lysates prepared from cells treated as *A*. The blots were probed with indicated antibodies. *C* and *D*, cisplatin-resistant A2780CP cells transfected, treated, and analyzed as described in *A* and *B* except LY294002 treatment. *E*, Tunel assay. A2780S cells were transfected with indicated plasmids and treated with cisplatin. All the experiments were performed in triplicate.

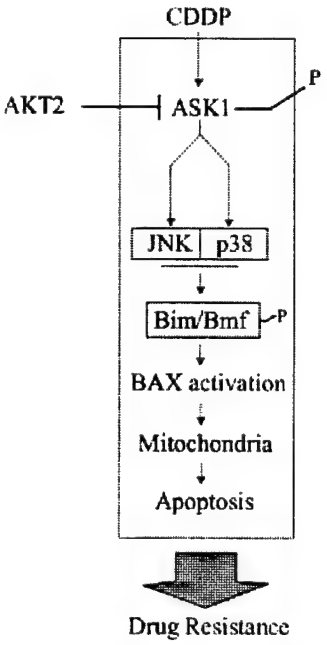


FIG. 8. **Schematic illustration of AKT2 regulation of ASK1/JNK/p38 and Bax.**

Besides the direct inhibition of ASK1, AKT2 could regulate JNK and p38 through other mechanisms. For example, NFκB-induced X chromosome-linked inhibitor of apoptosis and GADD45β down-regulate TNFα-induced JNK signaling (43, 44). We have demonstrated previously (21) that AKT2 inhibits UV- and TNFα-induced JNK and p38 by activation of the NFκB pathway (21). Therefore, we examined the possibility of AKT2 up-regulation of X chromosome-linked inhibitor of apoptosis and GADD45β. Western and Northern blot analyses, however, revealed no difference in X chromosome-linked inhibitor of apoptosis and GADD45β expression in A2780S cells transfected with constitutively active AKT2 or the control plasmid, pcDNA3 (data not shown). The possible reason is that cisplatin, unlike UV and TNFα, is incapable of inducing the NFκB pathway in A278S cells. In fact, our reporter assay revealed that cisplatin inhibits rather than activates NFκB activity in A2780S cells (data not shown).

In the present study, we observed that the ability of AKT2 to inhibit cisplatin-induced JNK/p38 was attenuated by nonphosphorylatable ASK1-S83A. Expression of phosphomimic ASK1-S83D alone was sufficient to inhibit JNK/p38 activation (Fig. 4). In addition, ASK1-S83D exhibited effects similar to that of constitutively active AKT2, *i.e.* rendered cells resistant to cisplatin, whereas ASK1-S83A sensitized cells to cisplatin-induced apoptosis (Fig. 7*E*). Thus, we conclude that AKT2 inhibition of cisplatin-stimulated JNK/p38 activation leading to cisplatin resistance is mediated by AKT2 phosphorylation/inhibition of ASK1.

It has been demonstrated that cisplatin-induced Bax conformational change is also important for cisplatin-stimulated apoptosis (45). Bax is a pro-apoptotic member of the Bcl2 family. Accumulated evidence shows that death signals, including cisplatin, induce a conformational change of Bax, leading to its mitochondrial translocation, oligomerization or cluster formation, and cytochrome *c* release (46, 47). Recent studies from Bax and/or Bak knock-out cells have shown that BH3-only proteins, such as tBid, Bad, Puma, and Bim, are required for inducing the activation of Bax and Bak by their direct interaction (48). Moreover, Akt has been shown to effectively inhibit Bax conformational change and contribute to chemoresistance (49). However, the mechanism by which Akt blocks Bax activation is poorly documented. We demonstrate in this report that ASK1 mediates at least in part cisplatin-induced Bax conformational change. Ectopic expression of constitutively active AKT2 attenuates cisplatin-induced Bax activation by phosphorylation and inhibition of ASK1. Downstream targets of ASK1, JNK, and p38, especially JNK, mediate AKT2 inhibition of Bax conformational change. These results are consistent with the recent findings obtained from a *Jnk*-deficient cell model (39).

Accumulated evidence shows that AKT2 plays a more significant role in human oncogenesis than AKT1 and AKT3. Frequent alterations of AKT2, but not AKT1 and AKT3, were detected in human cancers (18). Further, ectopic expression of AKT2, but not AKT1 and AKT3, leads to increased invasion and metastasis of human breast and ovarian cancer cells (50) and to malignant transformation of mouse fibroblasts (19). We observed in this study that A2780S cells expressing constitutively active AKT2 became cisplatin-resistant whereas expression of dominant negative AKT2 or treatment with PI3K inhibitor sensitized both cisplatin-sensitive (A2780S) and -resistant (A2780CP) ovarian cancer cells to cisplatin-induced apoptosis. Moreover, cisplatin-induced programmed cell death was enhanced by the expression of AKT2 nonphosphorylatable ASK1-S83A, whereas it is inhibited by phosphomimic ASK1-S83D. These data, therefore, indicate that activation of AKT2 contributes to cisplatin resistance by regulation of the ASK1/JNK/p38/Bax pathway and that the PI3K/AKT2/ASK1 cascade could be a critical therapeutic target for human cancer (Fig. 8).

A recent report (51) demonstrates that JNK and p38 phosphorylate BH3-only proapoptotic proteins Bim and Bmf, which was thought to mediate UV-induced apoptosis through a Bax-dependent mitochondrial apoptotic pathway (Fig. 8). Further investigation is required to determine the molecular mechanism by which ASK1/JNK/p38 regulates Bax activation in ovarian cancer cells, *i.e.* whether ASK1 and/or cisplatin induce Bim and Bmf phosphorylation and whether the phosphorylation is inhibited by PI3K/AKT2 pathway.

Acknowledgments—We are grateful to Hidenori Ihijo for pcDNA$_3$-HA-ASK1, Roger Davis for JNK and p38 plasmids, and Benjamin K. Tsang for ovarian cancer cell lines. We are also grateful to the DNA Sequence Facility at H. Lee Moffitt Cancer Center for sequencing ASK1 mutant expression constructs.

REFERENCES

1. Charlotte, M., and Stan, B. K. (2002) *Eur. J. Cancer* **38**, 1701–1707
2. Ozoles, R. F., and R. C. Young. (1991) *Semin. Oncol.* **11**, 251–263
3. Hamilton, T. C., Winker, M. A., Louie, K. G., Batist, G., Behrens, B. C., Tsuruo, T., Grotzinger, K. R., McKoy, W. M., Young, R. C., and Ozols, R. F. (1985) *Biochem. Pharmacol.* **34**, 2583–2586
4. Godwin, A. K., Meister, A., O'Dwyer, P. J., Huang, C. S., Hamilton, T. C., and Anderson, M. E. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 3070–3074
5. Parker, R. J., Eastman, A., Bostic-Burton, F., and Reed, E. (1991) *J. Clin. Invest.* **87**, 772–777
6. Fujiwara, T., Grimm, E. A., Mukhopadhyay, T, Zhang, W. W., Owen-Schaub, L. B., and Roth, J. A. (1994) *Cancer Res.* **54**, 2287–2291
7. Vikhanskaya, F., Erba, E., D'Incalci, M., and Broggini, M. (1994) *Nucleic Acids Res.* **22**, 1012–1017
8. Cantley, L. C. (2002) *Science* **296**, 1655–1657
9. Franke, T. F., Yang, S. L., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Tsichlis, P. N. (1995) *Cell* **81**, 727–736
10. Burgering, B. M. T., and Coffer, P. J. (1995) *Nature* **376**, 599–602
11. Shaw, M., Cohen, P., and Alessi, D. R. (1998) *Biochem. J.* **336**, 241–246
12. Liu, A.-X., Testa, J. R., Hamilton, T. C., Jove, R., Nicosia, S. V., and Cheng, J. Q. (1998) *Cancer Res.* **58**, 2973–2977
13. Alessi, D. R., and Cohen, P. (1998) *Curr. Opin. Genet. Dev.* **8**, 55–62
14. del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, C. R., and Nunez, G. (1997) *Science* **278**, 687–689
15. Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C. (1998) *Science* **282**, 1318–1321
16. Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1999) *Cell* **96**, 857–868
17. Cheng, J. Q., Godwin, A. K., Bellacosa, A., Taguchi, T., Franke, T. F., Hamilton, T. C., Tsichlis, P. N., and Testa, J. R. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 9267–9271
18. Yuan, Z. Q., Sun, M., Feldman, R. I., Wang, G., Ma, X., Jiang, C., Coppola, D., Nicosia, S. V., and Cheng, J. Q. (2000) *Oncogene* **19**, 2324–2330

19. Cheng, J. Q., Altomare, D. A., Klein, M. A., Lee, W. C., Kruh, G. D., Lissy, N. A., and Testa, J. R. (1997) *Oncogene* **14,** 2793–2801
20. Jiang, K., Coppola, D., Crespo, N. C., Nicosia, S. V., Hamilton, A. D., Sebti, S. M., and Cheng, J. Q. (2000) *Mol. Cell. Biol.* **20,** 139–148
21. Yuan, Z. Q., Feldman, R. I., Sun, M., Olashaw, N. E., Coppola, D., Sussman, G. E., Shelley, S. A., Nicosia, S. V., and Cheng, J. Q. (2002) *J. Biol. Chem.* **277,** 29973–29982
22. Benhar, M., Dalyot, I., Engelberg, D., and Levitzki, A. (2001) *Mol. Cell. Biol.* **21,** 6913–6926
23. Sanchez-Perez, I., Murguia, J. R., and Perona, R. (1998) *Oncogene* **16,** 533–540
24. Tibbles, L. A., and Woodgett, J. R. (1999) *Mol. Life Sci.* **55,** 1230–1254
25. Yamamoto, K., Ichijo, H., and Korsmeyer, S. J. (1999) *Mol. Cell. Biol.* **19,** 8469–8478
26. Ichijo, H., Nishida, E., Irie, K., ten Dijke, P., Saitoh, M., Moriguchi, T., Takagi, M., Matsumoto, K., Miyazono, K., and Gotoh, Y. (1997) *Science* **275,** 90–94
27. Nishitoh, H., Saitoh, M., Mochida, Y., Takeda, K., Nakano, H., Rothe, M., Miyazono, K., and Ichijo, H. (1998) *Mol. Cell* **2,** 389–395
28. Chang, H. Y., Nishitoh, H., Yang, X., Ichijo, H., and Baltimore, D. (1998) *Science* **281,** 1860–1863
29. Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., and Ichijo, H. (1998) *EMBO J.* **17,** 2596–2606
30. Gotoh, Y., and Cooper, J. A. (1998) *J. Biol. Chem.* **273,** 17477–17482
31. Wang, T. H., Wang, H. S., Ichijo, H., Giannakakou, P., Foster, J. S., Fojo, T., and Wimalasena, J. (1998) *J. Biol. Chem.* **273,** 4928–4936
32. Chen, Z., Seimiya, H., Naito, M., Mashima, T., Kizaki, A., Dan, S., Imaizumi, M., Ichijo, H., Miyazono, K., and Tsuruo, T. (1999) *Oncogene* **18,** 173–180
33. Tobiume, K., Matsuzawa, A., Takahashi, T., Nishitoh, H., Morita, K., Takeda, K., Minowa, O., Miyazono, K., Noda, T., and Ichijo, H. (2001) *EMBO Rep.* **2,** 222–228
34. Sun, M., Wang, G., Paciga, J. E., Feldman, R. I., Yuan, Z. Q., Ma, X. L., Shelley, S. A., Jove, R., Tsichlis, P. N., Nicosia, S. V., and Cheng, J. Q. (2001) *Am. J. Pathol.* **159,** 431–437
35. Asselin, E., Mills, G. B., and Tsang, B. K. (2001) *Cancer Res.* **61,** 1862–1868
36. Sugimoto, C., Fujieda, S., Seki, M., Sunaga, H., Fan, G. K., Tsuzuki, H., Borner, C., Saito, H., and Matsukawa, S. (1999) *Int. J. Cancer.* **82,** 860–867
37. Makin, G. W., Corfem B. M., Griffiths, G. J., Thistlethwaite, A., Hickman, J. A., and Dive, C. (2001) *EMBO J.* **20,** 6306–6315
38. Sanchez-Perez, I., and Perona, R. (1999) *FEBS Lett.* **453,** 151–158
39. Lei, K., Nimnual, A., Zong, W. X., Kennedy, N. J., Flavell, R. A., Thompson, C. B., Bar-Sagi, D., and Davis, R. J. (2002) *Mol. Cell. Biol.* **22,** 4929–4942
40. Wang, X., Martindale, J. L., Liu, Y., and Holbrook, N. J. (1998) *Biochem. J.* **333,** 291–300
41. Tournier, C., Hess, P., Yang, D. D., Xu, J., Turner, T. K., Nimnual, A., Bar-Sagi, D., Jones, S. N., Flavell, R. A., and Davis, R. J. (2000) *Science* **288,** 870–874
42. Kim, A. H., Khursigara, G., Sun, X., Franke, T. F., and Chao, M. V. (2001) *Mol. Cell. Biol.* **21,** 893–901
43. De Smaele, E., Zazzeroni, F., Papa, S., Nguyen, D. U., Jin, R., Jones, J., Cong, R., and Franzoso, G. (2001) *Nature* **414,** 308–313
44. Tang, G., Minemoto, Y., Dibling, B., Purcell, N. H., Li, Z., Karin, M., and Lin, A. (2001) *Nature* **414,** 313–317
45. Devarajan, P., Savoca, M., Castaneda, M. P., Park, M. S., Esteban-Cruciani, N., Kalinec, G., and Kalinec, F. (2002) *Hearing Res.* **174,** 45–54
46. Gross, A., Jockel, J., Wei, M. C., and Korsmeyer, S. J. (1998) *EMBO J.* **17,** 3878–3885
47. Bellosillo, B., Villamor, N., Lopez-Guillermo, A., Marce, S., Bosch, F., Campo, E., Montserrat, E., and Colomer, D. (2002) *Blood* **100,** 1810–1816
48. Marani, M., Tenev, T., Hancock, D., Downward, J., and Lemoine, N. R. (2002) *Mol. Cell. Biol.* **22,** 3577–3589
49. Yamaguchi, H., and Wang, H. G. (2001) *Oncogene* **20,** 7779–7786
50. Arboleda, M. J., Lyons, J. F., Kabbinavar, F. F., Bray, M. R., Snow, B. E., Ayala, R., Danino, M., Karlan, B. Y., and Slamon, D. J. (2003) *Cancer Res.* **63,** 196–206
51. Lei, K., and Davis, R. J. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 2432–2437

Phosphatidylinositol-3-OH Kinase/Akt and Survivin Pathways as Critical Targets for Geranylgeranyltransferase I Inhibitors Induced Apoptosis

Han C. Dan†, Domenico Coppola†‡, Mei Sun† Kun Jiang†‡¶, Andrew Hamilton§, Santo V. Nicosia†‡, Said M. Sebti‡¶*, Jin Q. Cheng†‡¶*

‡*Departments of Pathology*† *and Interdisciplinary Oncology and* ¶*Drug Discovery Program, University of South Florida College of Medicine and H. Lee Moffitt Cancer Center, Tampa, Florida 33612,* §*Yale University, Department of Chemistry, New Haven, Connecticut 06511.*

Running Title: GGTI inhibits PI3K/AKT and survivin pathways

Key Words: Akt, PI3K, survivin, GGTI, apoptosis, ovarian cancer

* Correspondence: JQ Cheng, Department of Pathology University of South Florida College of Medicine, 12901 Bruce B. Downs Blvd., MDC11, Tampa, FL 33612, USA. E-mail: jcheng@hsc.usf.edu or Saïd Sebti: Drug Discovery Program, H. Lee Moffitt Cancer Center and Research Institute, 12902 Magnolia Drive, MRC-DRDIS, Tampa, Florida 33612. E-mail: Sebti@moffitt.usf.edu.

Geranylgeranyltransferase I inhibitors (GGTIs) represent a new class of anti-cancer drugs that show promise in blocking tumor growth. However, the mechanism by which GGTIs inhibit tumor cell growth is still unclear. Here, we demonstrate that GGTI-298 and GGTI-2166 induce apoptosis in both cisplatin sensitive and resistant human ovarian epithelial cancer cells by inhibition of PI3K/AKT and survivin pathways. Following GGTI-298 or GGTI-2166 treatment, kinase levels of PI3K and AKT were decreased and survivin expression was significantly reduced. Ectopic expression of constitutively active AKT2 and/or surviving significantly rescue human cancer cells from GGTI-298-induced apoptosis. Previous studies have shown that Akt mediates growth factor-induced survivin, whereas p53 inhibits survivin expression. However, constitutively active AKT2 failed to rescue the GGTIs downregulation of survivin. Further, GGTIs suppress survivin expression and induce programmed cell death in both wild type p53 and p53-deficient ovarian cancer cell lines. These data indicate that GGTI-298 and GGTI-2166 induce apoptosis by targeting PI3K/AKT and survivin parallel pathways independent of p53. Due to the fact that upregulation of Akt and survivin as well as inactivation of p53 are frequently associated with chemoresistance, GGTI-298 and GGTI-2166 could be valuable agents to overcome anti-tumor drug resistance.

Introduction

Geranylgeranyltransferase I and farnesyltransferase inhibitors (GGTIs and FTIs) represent a new class of anti-cancer drugs that show promise in blocking tumor growth (Sebti et al., 2000). These compounds were originally designed to block lipid posttranslational modification of oncogenic Ras, which is essential for its function (Reiss et al., 1990; Kohl et al., 1993). Prenylation of small G proteins such as Ras, Rho, and Rac is critical to their cellular localization and function. Two types of prenyl transferases, farnesytransferase and geranylgeranyltransferase (GGTase), have been shown to catalyze protein prenylation. FTase catalyzes the transfer of farnesyl from farnesylpyrophosphate to a cysteine at the carboxyl terminus of proteins ending in CAAX where C is cysteine and A is an aliphatic amino acid, and X is methionine, serine, cysteine, or glutamine. GGTase I, on the other hand, transfers geranylgeranyl from geranylgeranylpyrophosphate to CAAX terminal sequences where X is leucine or isoleucine. We have developed CAAX peptidomimetics such as GGTI-298 and FTI-277 as highly selective inhibitors of GGTase I and FTase, respectively (Sebti et al., 1997). FTI-277 blocks potently oncogenic H-Ras processing and signaling. However, inhibition of the processing of K-Ras, the most prevalent form of mutated Ras in human tumors, becomes geranylgeranylated by GGTase I when FTase is inhibited. Therefore, both FTI-277 and GGTI-298 are required for inhibition of K-Ras processing in human tumor (Sebti et al., 1997). However, recent studies demonstrate that GGTI and FTI inhibit tumor cell survival and growth by targeting other signaling molecules (Zeng et al., 2003; Jiang et al., 2000; Sun et al., 1999).

Inhibitor of apoptosis proteins (IAPs) represent a conserved gene family that protects against programmed cell death induced by a variety of apoptotic stimuli (Deveraux et al., 1999). IAPs contain at least one BIR (baculovirus IAP repeat) domain that binds to caspases 3, 7 and 9 to inhibit their activities. Survivin is the smallest known IAP family protein and contains a single BIR domain with which it binds caspases and prevents caspase-induced apoptosis (Altieri et al., 2003). In addition, survivin also plays an important role in cell cycle control (Reed et al., 2001). Altered expression of survivin appears to be a common event associated with the pathogenesis of human cancer; survivin is overexpressed in many transformed cell lines and in common cancers, such as those of the ovary, lung, colon, liver, prostate and breast (Altieri et al., 2003; Reed et al., 2001). Reduced survivin expression causes apoptosis and sensitization to anticancer drugs, suggesting that survivin expression is important for cell survival or chemoresistance of certain carcinomas (Altieri et al., 2003; Tran et al., 2002).

Phosphatidylinositol-3-OH kinase/Akt is another major cell survival pathway that has been recently extensively studied (Brazil et al., 2002). PI3K is a heterodimer composed of a p85-regulatory and a p110-catalytic subunit and converts the plasma membrane lipid phosphatidylinositol-4-phosphate [PI(4)P1] and phosphatidylinositol-4,5-bisphosphate [PI(4,5)P2] to phosphatidylinositol-3,4,-bisphosphate [PI(3,4)P2] and phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P3]. Pleckstrin-homology (PH) domain-containing proteins, including Akt, accumulate at sites of PI3K activation by directly binding to PI(3,4)P2 and PI(3,4,5)P3. Akt (also known as PKB) represents a

subfamily of the serine/threonine kinases. Three members of this family, including AKT1, AKT2, and AKT3, have been identified so far. Akt is activated by a variety of stimuli, including growth factors, protein phosphatase inhibitors, and stress in a PI3K-dependent manner (Franke et al., 1995; Datta et al., 1999). Several downstream targets of Akt, each of which contains the Akt phosphorylation consensus sequence R-X-R-X-X-S/T-F/L, have been identified (Datta et al., 1999), pointing to the possible mechanisms by which Akt promotes cell survival and blocks apoptosis. Akt phosphorylates the proapoptotic proteins BAD, caspase-9, and transcription factor FKHRL1, resulting in reduced binding of BAD to Bcl-X_L and inhibition of caspase-9 protease activity and Fas ligand transcription (Datta et al., 1999). Moreover, alterations of Akt, especially AKT2, have been frequently detected in human malignancy. Overexpression/activation of PI3K and/or Akt renders cancer cells resistant to conventional chemotherapeutic drugs (Clark et al., 2002; Cheng et al., 2002). It has also been shown that inactivation of PTEN and p53 results in constitutive activation of Akt pathway. PTEN mutations lead to lost of its lipid phosphatase activity, and thus, it is unable to convert PI(3,4,5)P3 to PI(4,5)P2 (Datta et al., 1999). P53 transcription factor has recently been found to bind to the promoters of PTEN and p110α to induce PTEN and inhibit p110α transcription. Therefore, mutations of p53 result in downregulation of PTEN and upregulation of p110α leading to activation of Akt (Stambolic et al., 2001; Singh et al., 2002).

In the present study, we demonstrate that GGTI-298 and GGTI2166 target PI3K/AKT2 and survivin pathways leading to programmed cell death in cisplatin sensitive and resistant human ovarian cancer cells via a p53-independent mechanism. Moreover,

AKT1 activation was also inhibited by GGTI-298 and GGTI-2166. As AKT2, but not AKT1, is frequently altered in human cancer (Cheng et al., 1992; Cheng et al., 1996; Yuan et al., 2000; Arboleda et al., 2003), we primary focused our study on AKT2.

Results and Discussion

GGTIs Inhibit AKT2 and Induces Apoptosis in Cisplatin-Sensitive and Resistant Human Ovarian Cancer Cells

We have previously demonstrated that GGTI-298 arrests NIH 3T3 cells and lung cancer cells at G1 phase by upregulation of $p21^{WAF/CIP1}$ and hypophosphorylation of RB (Sun et al., 1999; Adnane et al., 1998). We have also documented that GGTIs enhance the ability of FTIs to induce apoptosis in drug-resistant myeloma (Xia et al., 2001) as well as synergize with other anticancer drugs such as cisplatin, taxol, and gemcitabine to inhibit human lung cancer cell growth in nude mice (Sun and Blaskovich et al., 1999). These results implicate the role of geranylgeranylated proteins in cell survival control, yet the involved mechanisms for inhibition of tumor growth and induction of apoptosis still remain unclear. Our previous studies showed that constitutively active H-Ras significantly activates PI3K/AKT2 and that the farnesyltransferase inhibitor, FTI-277, suppresses the PI3K/AKT2 pathway leading to cell death in human cancer cell lines (Jiang et al., 2000; Liu et al., 1998). These studies prompted us to examine the possible involvement of the PI3K/AKT2 pathway in GGTI anti-tumor activity. A cisplatin-sensitive (A2780S) and a cisplatin- resistant (A2780CP) ovarian cancer cell lines were

treated with GGTI-298 (15 μM) or GGTI-2166 (20 μM) in DMEM supplemented with 10% FBS for 0, 12, 24, 36 and 48 hours. Apoptosis and AKT2 activation were analyzed with Tunel assay and Western blot. Following GGTI-298 or GGTI-2166 treatment, both cisplatin-sensitive A2780S and cisplatin-resistant A2780CP cells underwent programmed cell death. Apoptotic cells reached approximately 70%-80% after 36 hours of treatment without significant difference between these two cell lines (Fig. 1A), indicating that GGTI-298 and GGTI-2166 are able to overcome cisplatin resistance in human ovarian cancer cells.

Immunoblotting analysis of AKT2 immunoprecipitates with phosphor-Akt-Ser473 antibody revealed that GGTI-298 and GGTI-2166 inhibits AKT2 phosphorylation after 12 hours of treatment in both cisplatin sensitive and resistant cell lines. However, total AKT2 protein levels remained unchanged (Fig. 1B). Moreover, immunoblotting analysis also showed that AKT1 phosphorylation was inhibited following GGTI treatment (data not shown). These results suggest that GGTI may either directly or indirectly target Akt signal transduction pathway to induce apoptosis.

GGTIs Target a Geranylgeranylated Protein(s) Upstream of PI3K/AKT2 Pathway

To demonstrate that GGTI-298 and GGTI-2166 actually suppress AKT2 kinase, A2780S cells were treated with or without EGF (50 ng/ml) for 15 min following treatment with GGTI-298 or GGTI-2166 for 12 hours. *In vitro* kinase assays were then performed on AKT2 immunoprecipitates as described under Experimental Procedures. As illustrated in

Fig. 2A, EGF-induced AKT2 kinase activity was abrogated by GGTI-298 treatment. As PI3K is an upstream activator of AKT2, we next examined whether GGTI-298 inhibits PI3K activity. Following GGTI-298 treatment and EGF stimulation as described above for AKT2 kinase assay, A2780S and A2780CP cells were immunoprecipitated with anti-pan-p85 antibody. PI3K activity was examined by *in vitro* kinase analysis of the immunoprecipitates using PI(4,5)P2 as a substrate. GGTI-298 attenuated EGF-stimulated PI3K activation (Fig. 2C). However, GGTI-298 does not directly inhibit PI3K and AKT2 activities as determined by adding GGTI-298 to the kinase reaction *in vitro* (Figs. 2B and 2D). In addition, GGTI-2166 exhibits the same effects on PI3K/Akt activation as GGTI-298 (data not shown). These data imply that GGTI-298 and GGTI-2166 are not direct inhibitors of PI3K and AKT2 but rather target a geranylgeranylated protein(s) upstream of PI3K/AKT2 pathway.

Constitutively Active AKT2 Partially Rescues A2780S Cells from GGTIs-induced Apoptosis

We reasoned that if GGTI-298 and GGTI-2166 inhibit a geranylgeranylated protein upstream of PI3K/AKT2, then constitutively active AKT2 should overcome GGTIs-induced apoptosis. A constitutively active AKT2 expression construct (HA-Myr-*AKT2*) or $pcDNA_3$ vector alone was stably transfected into A2780S cells. Western blot analysis with anti-HA antibody revealed expression of HA-Myr-AKT2 in the transfectants (Fig. 3A). After treatment with GGTI-298 (15 μM) or GGTI-2166 (20 μM) for different times in the presence of 10% FBS, apoptotic cells were observed in $pcDNA_3$- and Myr-AKT2-

transfected A2780S cells. The percentages of apoptotic cells in pcDNA3-transfected A2780S cells increased from 8% at time 0 to 80% after 48 hours of treatment with GGTI-298 (Fig. 3B). These percentages are very similar to those reported in Figure 1A for non-transfected parental A2780S cells. In contrast, GGTI-298 induced apoptosis by 40% at time 48 hours of treatment in cells transfected with constitutively activated AKT2. Similar effects were observed in the cells treated with GGTI-2166 (data not shown). Therefore, constitutively active AKT2 only partially rescues A2780S cells from GGTIs induced apoptosis (Fig. 3B), indicating that other cell survival signal molecule(s) must be targeted by GGTI-298 and GGTI-2166 besides PI3K/Akt pathway.

GGTIs Downregulate the IAP Family Protein, Survivin

Numerous studies have shown that IAP family proteins play a critical role in cell survival (Deveraux et al., 1999; Altieri et al., 2003; Reed et al., 2001; Tran et al., 2002). Among the members of IAP family, only survivin is frequently overexpressed in human cancer including ovarian carcinoma and ectopic expression of survivin renders ovarian cancer cells resistant to taxol (Zaffaroni et al., 2002). Thus, we next examined whether GGTI-298 or GGTI2166 target survivin to induce apoptosis in ovarian cancer cells. A2780S cells, in which survivin is highly expressed, were treated with GGTI-298 or GGTI-2166 for different times. Western and Northern blot analyses revealed that both protein and mRNA levels of survivin were significantly reduced following GGTI-298 or GGTI-2166 treatment (Fig, 4A and data not shown). To further examine the importance of survivin in GGTIs pro-apoptotic activity, A2780S cells were stably transfected with Myc-tagged

survivin. Again, the cells transfected with pcDNA3 vector alone were used as control. Expression of transfected Myc-survivin was confirmed by immunoblotting analysis with anti-Myc antibody (Fig. 4C). Following administration of GGTI-298 or GGTI-2166 at various lengths of time, apoptotic cells were detected by Tunel assay and quantified. Both GGTI-298 and GGTI-2166 increased apoptosis from 8% at time 0 to 70% at time 24 hours in A2780S-pcDNA3 cells. In survivin-expressing cells, both inhibitors induced apoptosis to only 30% after 24 hours of treatment (Fig. 4D and data not shown). Thus, ectopic expression of survivin rescues the cells from GGTIs induced apoptosis but only partially, implying that survivin is another target of GGTIs in addition to PI3K/AKT2. Further, A2780S cells were stably contransfected with constitutively active AKT2 and survivin (Fig. 4C) and treated with either GGTI-298 (15 μM) or GGTI-2166. Tunel assay analysis revealed that cells expressing both myr-AKT2 and surviving became dramatically resistant to GGTIs treatment (Fig. 4D), indicating that AKT2 and survivin are critical targets of GGTIs at least in A2780S ovarian cancer cells.

GGTIs Inhibits Survivin via a p53-independent Pathway

Previous investigations have demonstrated that p53 represses survivin expression through inhibiting its transcription (Hoffman et al., 2002; Mirza et al., 2002). To determine whether GGTIs suppression of survivin expression depends on p53, we evaluated the effects of GGTIs on survivin expression in A2780CP cells that carry p53 mutation (Sasaki et al., 2000). A2780CP cells were cultured in DMEM supplemented with 10% FBS and treated with GGTI-298 or GGTI-2166 for different times. The expression of

survivin was evaluated by Western and Northern blot analyses. Both protein and mRNA levels of survivin were inhibited by GGTI-298 and GGTI2166 treatment in A2780CP cells (Fig. 4B and data not shown). Quantification analysis showed that GGTIs-inhibited survivin expression was similar in A2780CP that contain mutant p53 and A2780S cells which express wild type p53 (Figs. 4A and 4B). To further define the effects of p53 on GGTIs suppression of survivin expression, A2780CP cells were stably transfected with HA-tagged wild type p53 and pcDNA3 vector alone, as a control. Fig. 5 A shows that transfected p53 expresses and is functional reflected by elevated level of $p21^{WAF1}$. Immunoblotting analysis showed that reintroduction of wild type p53 into A2780CP cells did not have significant effects on the ability of GGTIs to inhibit survivin expression as compared to pcDNA3-transfected A2780CP cells (Fig. 5B). These results indicate that GGTIs suppression of survivin is independent of p53 pathway.

Previous studies have shown that re-expression of wild type p53 sensitizes A2780CP cells to cisplatin-induced apoptosis (Sasaki et al., 2000; Song et al., 1997). Therefore, we next examined whether ectopic expression of wild type p53 sensitizes A2780CP cells to GGTIs stimulated cell death. Tunel assay revealed that the levels of GGTIs-induced apoptosis were the same in A2780CP-p53, A2780CP-pcDNA3 as well as A2780S cells (Figs. 5C and 1A). We have previously shown GGTI-298-mediated transcriptional upregulation of $p21^{WAF1/CIP1}$ is also independent of p53 (Adnane et al., 1998). This further supports the notion that the mechanism of GGTIs anti-tumor activity does not involve the p53 pathway. Because mutant p53 is a major contributor to anticancer drug resistance and because GGTIs can overcome this resistance at least in the

case of cisplatin, combination of GGTIs with these agents has great potential for cancer treatment.

GGTIs Attenuated AKT2-induced Survivin Expression and Promoter Activity

Recent studies have shown that PI3K/Akt pathway mediates IGF1- and VEGF-upregulation of survivin protein in multiple myeloma and endothelial cells (Mitsiades et al., 2002; Papapetropoulos et al., 2000). However, the underlying molecular mechanism has not been well documented. As GGTIs inhibits PI3K/AKT activation as well as survivin expression at the transcription level, we reasoned that activation of AKT2 could induce survivin transcription. To this end, Northern blot analysis of A2780S cells transfected with constitutively active AKT2 revealed that expression of survivin was induced by AKT2 in a dose dependent manner (Fig. 6A). Further, a luciferase activity assay was carried out with HEK293 cells transfected with pGL3-survivin-Luc reporter, constitutively active AKT2 and β-galactosidase. Triple experiments showed that ectopic expression of constitutively active AKT2 stimulated survivin promoter activity (Fig. 6B). These data indicate that AKT2 upregulates survivin by inducing its promoter activity. It has been demonstrated that survivin promoter contains a NFκB binding site and is induced by NFκB pathway (Deveraux et al., 1999; mitsiades et al., 2002). We and others have shown that AKT1 and AKT2 activate the NFκB pathway through interaction and phosphorylation of IKKα and Cot/Tpl2 (Ozes et al., 1999; Madrid et al., 2000; Yuan et al., 2002; Kane et al., 2002). Therefore, AKT2-induced survivin transcription could be mediated by activation of this pathway.

Since AKT2 upregulates survivin and GGTIs repress survivin expression and AKT2 activity, one possible mechanism by which GGTIs repress survivin is through inhibition of PI3K/AKT2. To test this hypothesis, constitutively active AKT2- and pcDNA3-stably transfected A2780S cells were treated with GGTI-298 or GGTI-2166. Following the treatment for 12, 24, and 48 hours, expression of survivin was examined by Western and Northern blot analyses. As shown in Fig. 6C, both basal protein and mRNA levels of survivin were higher in A2780S-Myr-AKT2 cells as compared to A2780S transfected with pcDNA3 vector alone. However, declining rate of the survivin induced by GGTIs was essentially the same between constitutively active AKT2- and pcDNA3-transfected A2780S cells. Moreover, the luciferase reporter assay showed that constitutively active AKT2-stimulated survivin promoter activity was also attenuated by GGTIs treatment. Even the basal levels of survivin promoter activity were significantly inhibited by GGTI-298 or GGTI-2166 (Fig. 6D and data not shown). Because GGTI-298 and GGTI-2166 are not direct AKT2 inhibitor (Fig. 2C), we conclude that GGTIs repress survivin by targeting other molecule(s) which bypasses AKT2 but is capable of blocking AKT2-induced survivin transcription. Moreover, these data also indicate that GGTIs induce apoptosis in human ovarian cancer cells by inhibition of survivin and PI3K/AKT2 parallel pathways.

In summary the data presented here demonstrate for the first time that GGTI-298 and GGTI-2166 potently inhibit PI3K/AKT2 activation and survivin expression in both cisplatin sensitive and resistant human ovarian cancer cell lines. Furthermore, our data

suggest a mechanism by which GGTIs repress survivin expression by showing that GGTI-298 inhibits mRNA and promoter activity of survivin independent of p53 status and AKT2 activation (Fig. 7). Finally we provide evidence that GGTIs-induced apoptosis is independent of p53 pathway and thus, GGTIs could be a new and potent therapeutic reagent to overcome p53 mutation-related chemoresistance. Further investigations are required to characterize the mechanism by GGTIs downregulate survivin and inactivate PI3K, i.e., identification of GGTI-298- and GGTI-2166-targeted geranylgeranylated proteins that positively regulate PI3K/Akt and survivin pathways independently (Fig. 7).

Material and Methods

Cell lines, Transfection, and Cell Treatment

Human ovarian epithelial cancer cell lines A2780S and A2780CP and human embryonic kidney (HEK) 293 were cultured at 37°C and 5% CO2 in DMEM supplemented with 10% FBS. The cells were seeded in 60-mm petri dishes at a density of 0.6 x 10^6 cells/dish and were transfected with 2 μg of DNA per dish using LipofecAMINE Plus. Stable clonal cell lines were established by G418 (500 ug/ml) selection.

Expression Constructs

HA-AKT2 and HA-Myr-AKT2 were prepared as described previously (Jiang et al., 2000). HA-tagged p53 was prepared by releasing p53 from GST-p53 plasmid, kindly provided by Jiandong Chen at H. Lee Moffitt Cancer Center, and cloning to HA-pcDNA3.1 vector. Survivin expression plasmid was created by PCR, subcloned to Myc-tagged pcDNA3.1 and confirmed by sequencing analysis. Based on published sequence (Li et al., 1999), survivin promoter (-1469/+20) was amplified by PCR using normal human placenta genomic DNA as template. The PCR products were ligated into *Bam*HI-*Sma*I sites of pGL3 vector. The promoter sequence was confirmed by DNA sequencing.

Tunel Assay

Cells were seeded into 60 mm dishes and grown in 10% FBS-DMEM for 36 hours. Cells were then treated with 15 μM GGTI-298 for different times ranging from 0 to 48 hours. Apoptosis was determined by terminal Tunel assay using an *in situ* cell death detection kit (Boehringer Mannheim, Indianapolis, IN). The cells were trypsinized, and cytospin preparations were obtained. Cells were fixed with freshly prepared paraformaldehyde (4% in PBS, pH 7.4). Slides were rinsed with PBS, incubated in permeabilization solution, followed by Tunel reaction mixture for 60 min at 37°C in a humidified chamber. After a rinse, the slides were incubated with converter-alkaline phosphatase solution for 30 min at 37°C and then detected with alkaline phosphatase substrate solution (Vector

Laboratories, Burlingame, CA). After an additional rinse, the slides were mounted and analyzed under a light microscope. These experiments were performed in triplicate.

Immunoprecipitation, in vitro Kinase Assay, Western and Northern Blotting Analysis

Following stimulation and treatment with GGTI, cells were lysed and immunoprecipitated with anti-AKT2 or Ant-HA antibody. The immunprecipitates were subjected to *in vitro* kinase assay using histone H2B as substrate. Protein expression was determined by probing Western blots with the appropriate antibodies. For the detection of endogenous phospho-AKT2, Western blot analysis of the AKT2 immunoprecipitates was performed and detected with anti-phospho-Akt-Ser473 antibody. Detection of antigen-bounded antibody was carried out with the ECL Western Blotting Analysis System (Amersham). Northern blot was performed as previously described (Cheng et al., 1992).

PI3K Assay

PI3K was immunoprecipitated from the cell lysates with anti-pan-p85 antibody (Santa Cruz Biotechnology). The immunoprecipitates were washed once with cold PBS, twice with 0.5 M LiCl/0.1 M Tris (pH 7.4), and finally with 10 mM Tris/100 mM NaCl/1 mM EDTA. The presence of PI3K activity in immunoprecipitates was determined by incubating the beads in reaction buffer (10 mM HEPES [pH 7.4], 10 mM $MgCl_2$, 50 μM ATP) containing 20 μCi [γ-^{32}P] ATP and 10 μg L-α-phosphatidylinositol 4, 5-bis phosphate (Biomol) for 20 min at 25^{0}C. The reactions were stopped by adding 100 μl of

1 M HCl. Phospholipids were extracted with 200 µl $CHCl_3$/MeOH and phosphorylated products were separated by thin-layer chromatography as previously described (Jiang et al., 2000). The conversion of PI $(4,5)P_2$ to PI$(3,4,5)P_3$ was detected by autoradiography.

Luciferase Reporter Assay

Cells were seeded in 6-well plate and transfected with survivin-Luc reporter, pSV2-β-gal and, constitutively active of AKT2. After 36 hours of transfection and treatment with or without GGTI-298, luciferase and β-galactosidase assays were performed according to the manufacturer's procedures (Promega and Tropix), respectively. Each experiment was repeated three times.

Acknowledgements

We are grateful to Jian-dong Chen for GST-p53 plasmid and Benjamin K. Tsang for ovarian cancer cell lines. We are also grateful to the Molecular Biology DNA Sequence Facility at H. Lee Moffitt Cancer Center for sequencing Survivin constructs. This work was supported by National Cancer Institute Grants CA085709, CA67771, CA89242 and Department of Defense DAMD 17-01-1-0394 and DAMD17-02-1-0671.

REFERENCES

Adnane J, Bizouarn FA, Qian Y, Hamilton AD and Sebti SM. (1998). *Mol. Cell. Biol.,* **18,** 6962-6970.

Altieri DC. (2003). *Nat. Rev. Cancer,* **3,** 46-54.

Arboleda MJ, Lyons JF, Kabbinavar FF, Bray MR, Snow BE, Ayala R, Danino M, Karlan BY and Slamon DJ. (2003). *Cancer Res,* 63,196-206.

Brazil DP, Park J and Hemmings BA. (2002). *Cell,* **111,** 293-303.

Cheng JQ, Jiang X, Fraser M, Li M, Dan HC, Sun M and Tsang BK. (2002). *Drug Resist. Updat,* **5,** 131-46.

Cheng JQ, Godwin AK, Bellacosa A, Taguchi T, Franke TF, Hamilton TC, Tsichlis PN and Testa JR. (1992). *Proc. Natl. Acad. Sci. U. S. A.,* **89,** 9267-9271.

Cheng JQ, Ruggeri B, Klein WM, Sonoda G, Altomare DA, Watson DK and Testa JR. (1996). *Proc. Natl. Acad. Sci. U. S. A.,* **93,** 3636-3641.

Clark AS, West K, Streicher S and Dennis PA. (2002). *Mol. Cancer Ther.,* **1,** 707-717.

Datta SR, Brunet A and Greenberg ME. (1999). *Genes Dev.,* **13,** 2905-2927.

Deveraux QL and Reed JC. (1999). *Genes Dev.,* **13,** 239-252.

Franke TF, Yang SL, Chan TO, Datta K, Kazlauskas A, Morrison DK, Kaplan DR, and Tsichlis PN. (1995). *Cell,* **81**, 727–736.

Hoffman WH, Biade S, Zilfou JT, Chen J and Murphy M. (2002). *J. Biol. Chem.,* **277,** 3247-3257.

Jiang K, Coppola D. Crespo NC. Nicosia SV, Hamilton AD, Sebti SM and Cheng JQ. (2000). *Mol. Cell. Biol.,* **20,**139-148.

Kane LP, Mollenauer MN, Xu Z, Turck CW, Weiss A. (2002). *Mol. Cell. Biol.,* **22,** 5962-5974.

Kohl NE, Mosser SD, deSolms SJ, Giuliani EA, Pompliano DL, Graham SL, Smith RL, Scolnick EM, Oliff A and Gibbs JB. (1993). *Science,* **260,** 1934-1937.

Li F, Altieri DC. (1999). *Biochem. J.,* **344,** 305-311.

Liu AX, Testa JR, Hamilton TC, Jove R, Nicosia SV and Cheng JQ. (1998). *Cancer Res.,* **58,** 2973-2977.

Madrid, L. V., Wang, C. Y., Guttridge, D. C., Schottelius, A. J., Baldwin, A. S. Jr., and Mayo, M. W. (2000) *Mol. Cell. Biol.* 20, 1626-1638

Mirza A, McGuirk M, Hockenberry TN, Wu Q, Ashar H, Black S, Wen SF, Wang L, Kirschmeier P, Bishop WR and Nielsen LL. (2002). *Oncogene,* **21,** 2613-2622.

Mitsiades CS, Mitsiades N, Poulaki V, Schlossman R, Akiyama M, Chauhan D, Hideshima T, Treon SP, Munshi NC, Richardson PG and Anderson KC. (2002). *Oncogene,* **21,** 5673-5683.

Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM and Donner DB. (1999). *Nature,* **401,** 82-85.

Papapetropoulos A, Fulton D, Mahboubi K, Kalb RG, O'Connor DS, Li F, Altieri DC and Sessa WC. (2000). *J. Biol. Chem.,* **275,** 9102-9105.

Reed JC. (2001). *J Clin Invest.,* **108,** 965-969.

Reiss Y, Goldstein JL, Seabra MC, Casey PJ and Brown MS. (1990). *Cell,* **62,** 81-88.

Sasaki H, Sheng Y, Kotsuji F and Tsang BK. (2000). *Cancer Res.,* **60,** 5659-5666.

Sebti SM and Hamilton AD. (1997). *Pharmacol. Ther.*, **74,** 103-114.

Sebti SM and Hamilton AD. (2000). *Oncogene,* **19,** 6584-6593.

Singh B, Reddy PG, Goberdhan A, Walsh C, Dao S, Ngai I, Chou TC, O-Charoenrat P, Levine AJ, Rao PH and Stoffel A. (2002). *Genes Dev.*, **16,** 984-93.

Song K, Li Z, Seth P, Cowan KH and Sinha BK. (1997). *Oncol. Res.*, **9,** 603-609.

Stambolic V, MacPherson D, Sas D, Lin Y, Snow B, Jang Y, Benchimol S and Mak TW. (2001). *Mol. Cell,* **8,** 317-325.

Sun J, Blaskovich MA, Knowles D, Qian Y, Ohkanda J, Bailey RD, Hamilton AD and Sebti SM. (1999). *Cancer Res.*, **59,** 4919-4926.

Sun J, Qian Y, Chen Z, Marfurt J, Hamilton AD and Sebti SM. (1999). *J. Biol. Chem.*, **274,** 6930-6934.

Tran J, Master Z, Yu JL, Rak J, Dumont DJ and Kerbel RS. (2002). *Proc. Natl. Acad. Sci. U. S. A.*, **99,** 4349-4354.

Xia Z, Tan MM, Wong WW, Dimitroulakos J, Minden MD and Penn LZ. (2001). *Leukemia,* **15,** 1398-1407.

Yuan ZQ, Feldman RI, Sun M, Olashaw NE, Coppola D, Sussman GE, Shelley SA, Nicosia SV and Cheng JQ. (2002). *J. Biol. Chem.*, **277,** 29973-3982.

Yuan Z, Sun M, Feldman RI, Wang G, Ma X, Coppola D, Nicosia SV and Cheng JQ. (2000). *Oncogene,* **19,** 2324-2330.

Zaffaroni N, Pennati M, Colella G, Perego P, Supino R, Gatti L, Pilotti S, Zunino F and Daidone MG. (2002). *Cell Mol. Life Sci.*, **59,** 1406-1412.

Zeng PY, Rane N, Du W, Chintapalli J and Prendergast GC. (2003). *Oncogene,* **22,** 1124-34.

FIGURE LEGENDS

Fig. 1. GGTI-298 inhibits AKT2 activation and induces apoptosis in cisplatin sensitive and resistant ovarian cancer cells. (**A**) Tunel assay. Cisplatin sensitive A2780-S and cisplatin resistant A2780-CP cells were cultured in DMEM supplemented with 10% FBS and treated with GGTI-298 (15 μM) for the indicated time. Apoptotic cells were detected with Tunel assay and quantified. (**B**) Western blot analyses of the AKT2 immunoprecipitates prepared from A2780S and A2780CP cells following GGTI-298 treatment. The blots were detected with anti-phospho-Akt-Ser473 (panels 1 and 3) and anti-AKT2 (panels 2 and 4) antibodies.

Fig. 2. GGTI-298 inhibits PI3K and AKT2 activation. (**A**) *In vitro* kinase assay of the HA-AKT2 immunoprecipitates prepared from A2780S cells. After serum starvation overnight, the cells were treated with or without GGTI-298 for 12 h prior to EGF (50 ng/ml) stimulation for 15 min. Immunoprecipitation was performed with anti-AKT2 antibody and subjected to *in vitro* kinase assay using histone H2B as substrate. (**B**) GGTI-298 does not directly inhibit EGF-induced AKT2 activation. After serum starvation and stimulation with EGF, GGTI-298 (15 μM) was directly added into AKT2 kinase reaction. Following incubation for 30 min, the reactions were separated on SDS-PAGE gel and exposed to the film. (**C**) *In vitro* PI3K assay of the anti-p85 immunoprecipitates prepared from A2780CP cells. Following serum starvation overnight, the cells were treated with or without GGTI-298 for 12 h prior to EGF stimulation for 15 min. (**D**) GGTI-298 does not directly inhibit EGF-induced PI3K

activation. *In vitro* PI3K assay of the PI3K immunoprecipitates derived from A2780CP cells. After serum starvation and stimulation, GGTI-298 (15 μM) was directly added to the kinase reaction. Quantification of AKT2 and PI3K activity is shown in bottom panels (A-D).

Fig. 3. A constitutively activated form of AKT2 partially rescues A2780S cells from GGTI-298-induced apoptosis. (**A**) A2780S cells were stably transfected with constitutively active AKT2 (Myr-AKT2, AA2). Western blot analysis with anti-HA antibody revealed expression of transfected HA-Myr-AKT2 in a clonal cell line (upper panel). Bottom panel shows equal loading. (**B**) Tunnel assay. After treatment of A2780S-pcDNA3 and A2780S-AA2 cells with GGTI-298 for the indicated times, apoptotic cells were detected with Tunel assay and quantified.

Fig. 4. GGTI-298 inhibits expression of survivin independent of p53 pathway. (**A** and **B**) Western (upper panels) and Northern (lower panels) analyses of expression of survivin in A2780S (wild type p53) and A2780CP (mutant p53) cells treated with GGTI-298 at indicated time. Northern blot analysis with [^{32}P]-dCTP labeled survivin cDNA probe (upper). Equal loading of total RNA was shown in bottom panel. (**C**) Immunoblotting analysis of expression of transfected Myc-survivin and HA-myr-AKT2 in A2780S cells with anti-Myc (upper) and anti-HA (bottom) antibodies. (**D**) Tunel assay. Following treatment of A2780S-pcDNA3, A2780S-survivin and A2780S-survivin/myr-AKT2 cells with GGTI-298 at indicated time, apoptotic cells were detected with Tunel assay and quantified.

Fig. 5. Ectopic expression of p53 did not affect GGTI action. (**A**) Immunoblotting analysis of expression of transfected wild type HA-p53 in A2780CP cells with anti-HA (top) and anti-p21 (middle) antibodies . The bottom panel showed equal loading. (**B**) Immunoblotting analysis of survivin expression in pcDNA3- (upper panels) and HA-p53-transfected (bottom panels) A2780CP cells. (**C**) Reintroduction of wild type p53 into A2780CP cells did not sensitize the cells to GGTI-298-induced apoptosis. Following GGTI-298 treatment at indicated time, apoptotic cells were detected with Tunel assay and quantified.

Fig. 6. Constitutively active AKT2 induces survivin transcription and promoter activity; AKT action failed to rescue GGTI downreulated survivin. (**A**) Northern blot analysis of A2780S cells transfected with indicated amount of constitutively active AKT2. The blot was probed with [^{32}P]-dCTP labeled survivin cDNA (upper panel). Equal loading was shown in panel 2. Expression of transfected constitutively active AKT2 was detected with anti-HA antibody (panel 3). The same blot was reprobed with anti-actin antibody (bottom panel). (**B**) Luciferase reporter assay. HEK293 cells were transfected with indicated plasmids. After 36 h of the transfection, luciferase and β-galactosidase assays were performed and the reporter activity was normalized by dividing luciferase activity with β-galactosidase. (**C**) Western (panels 1 and 2) and Northern (panels 3 and 4) blot analyses of pcDNA3- and constitutively active AKT2-trnasfected A2780S cells following treatment with GGTI-298 at indicated time. Western blots were detected with anti-survivin (upper) and anti-actin antibodies (panel 2). Northern blots were probed with

[^{32}P]-dCTP labeled survivin (panel 3). Equal RNA loading was shown in bottom panels. **(D)** Luciferase reporter assay was performed as described in Fig. 6B, except the cells were treated with indicated concentrations of GGTI-298 for 6 h prior to assay for luciferase and β-galactosidase activity.

Fig. 7. Schematic illustration of the mechanism of GGTI-298 induction of apoptosis in human cancer cells.

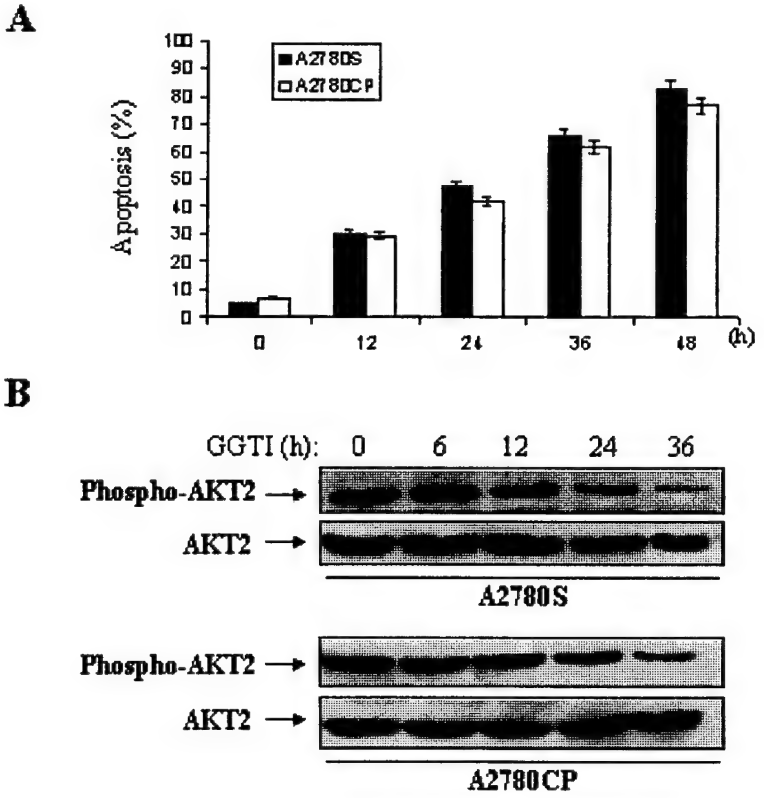

A
A2780S
A2780CP
Apoptosis (%)
100
90
80
70
60
50
40
30
20
10
0
0
12
24
36
48
(h)
B
GGTI (h): 0 6 12 24 36
Phospho-AKT2
AKT2
A2780S
Phospho-AKT2
AKT2
A2780CP

Fig. 1

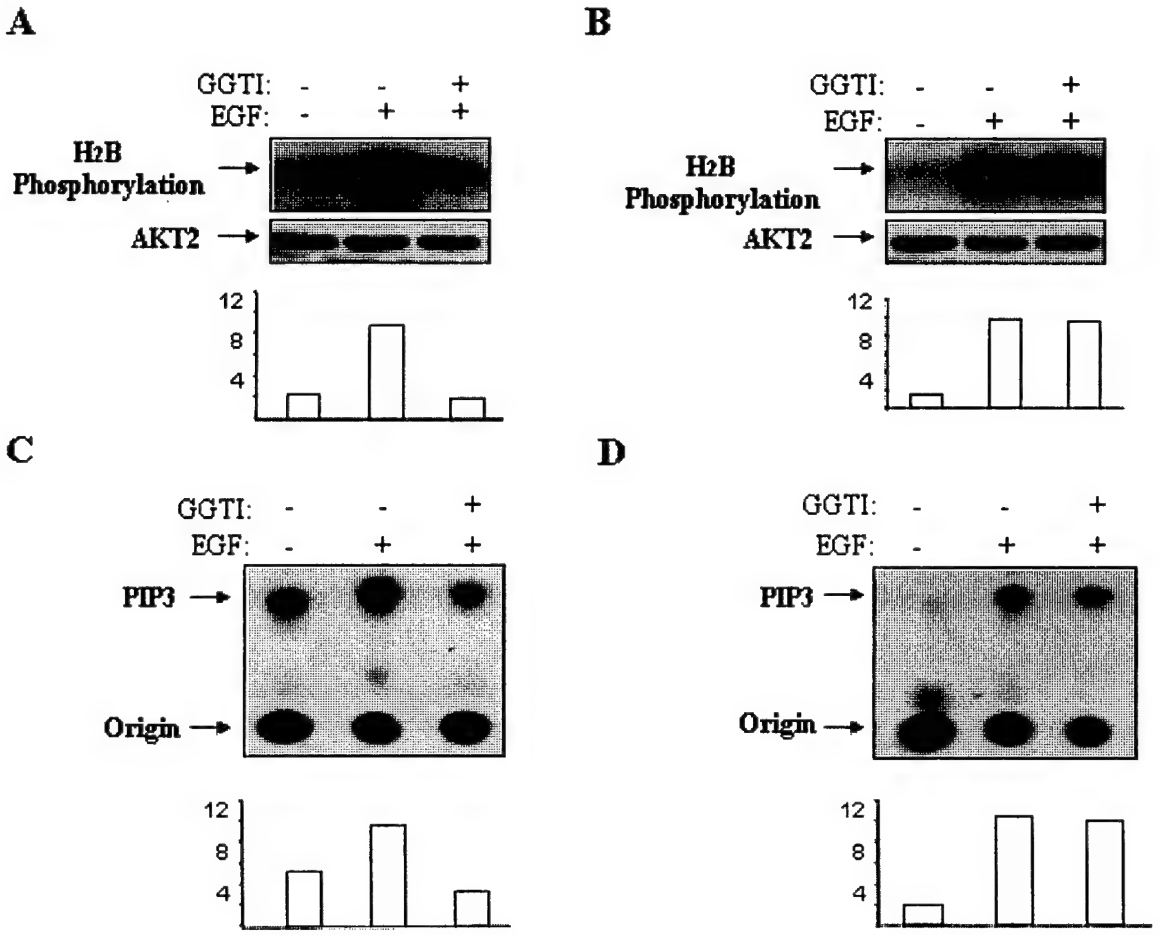
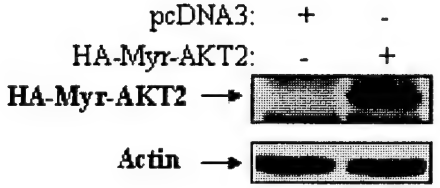

A
GGTI: - - +
EGF: - + +
H2B Phosphorylation
AKT2
12
8
4
B
GGTI: - - +
EGF: - + +
H2B Phosphorylation
AKT2
12
8
4
C
GGTI: - - +
EGF: - + +
PIP3
Origin
12
8
4
D
GGTI: - - +
EGF: - + +
PIP3
Origin
12
8
4

Fig. 2

A


pcDNA3: + -
HA-Myr-AKT2: - +
HA-Myr-AKT2
Actin

B

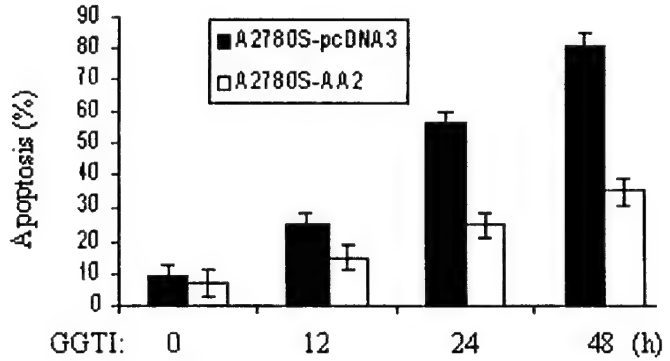

A2780S-pcDNA3
A2780S-AA2
Apoptosis (%)
90
80
70
60
50
40
30
20
10
0
GGTI: 0 12 24 48 (h)

Fig. 3

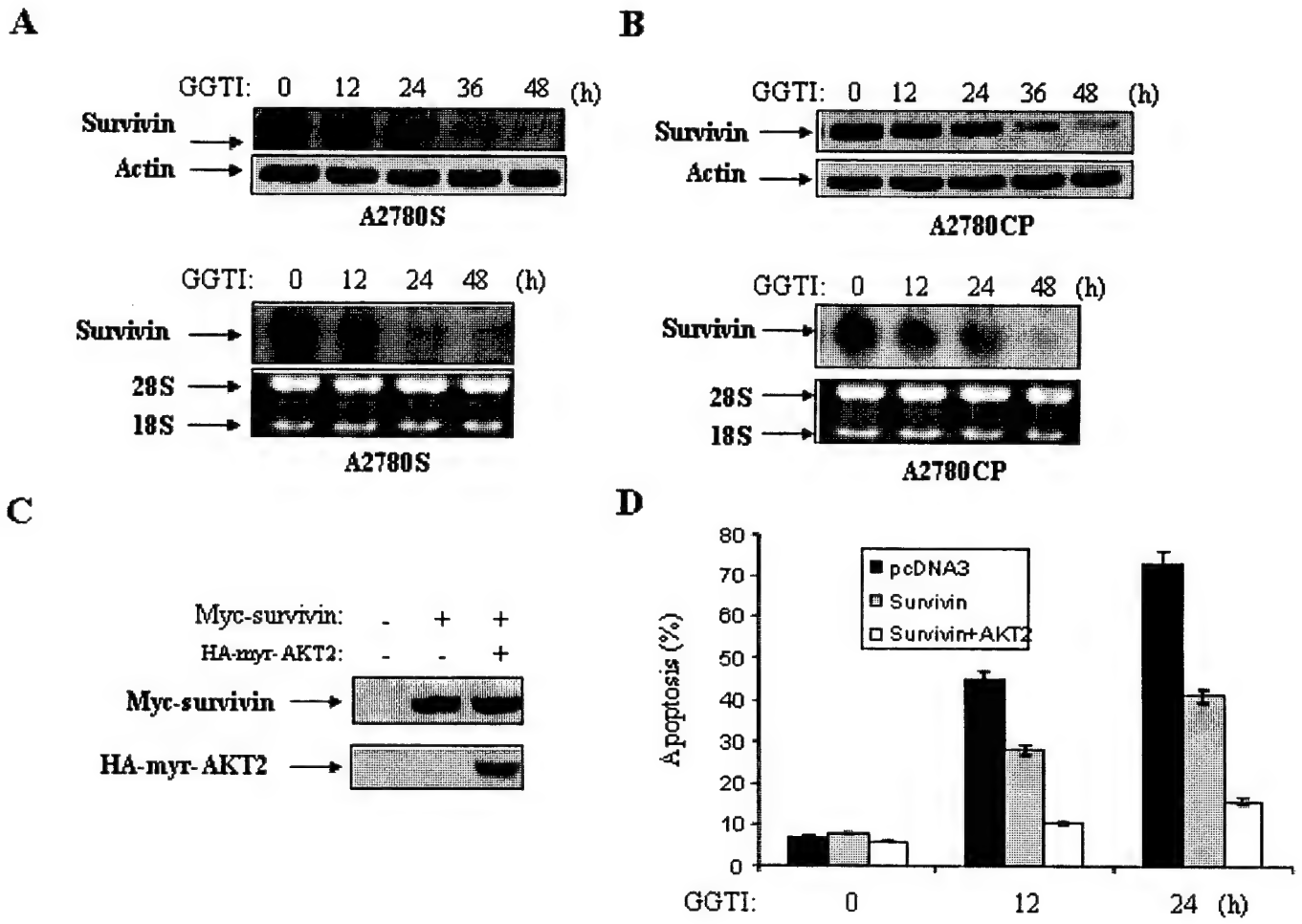

A
GGTI: 0 12 24 36 48 (h)
Survivin
Actin
A2780S
GGTI: 0 12 24 48 (h)
Survivin
28S
18S
A2780S
B
GGTI: 0 12 24 36 48 (h)
Survivin
Actin
A2780CP
GGTI: 0 12 24 48 (h)
Survivin
28S
18S
A2780CP
C
Myc-survivin: - + +
HA-myr-AKT2: - - +
Myc-survivin
HA-myr-AKT2
D
Apoptosis (%)
80
70
60
50
40
30
20
10
0
pcDNA3
Survivin
Survivin+AKT2
GGTI: 0 12 24 (h)

Fig. 4

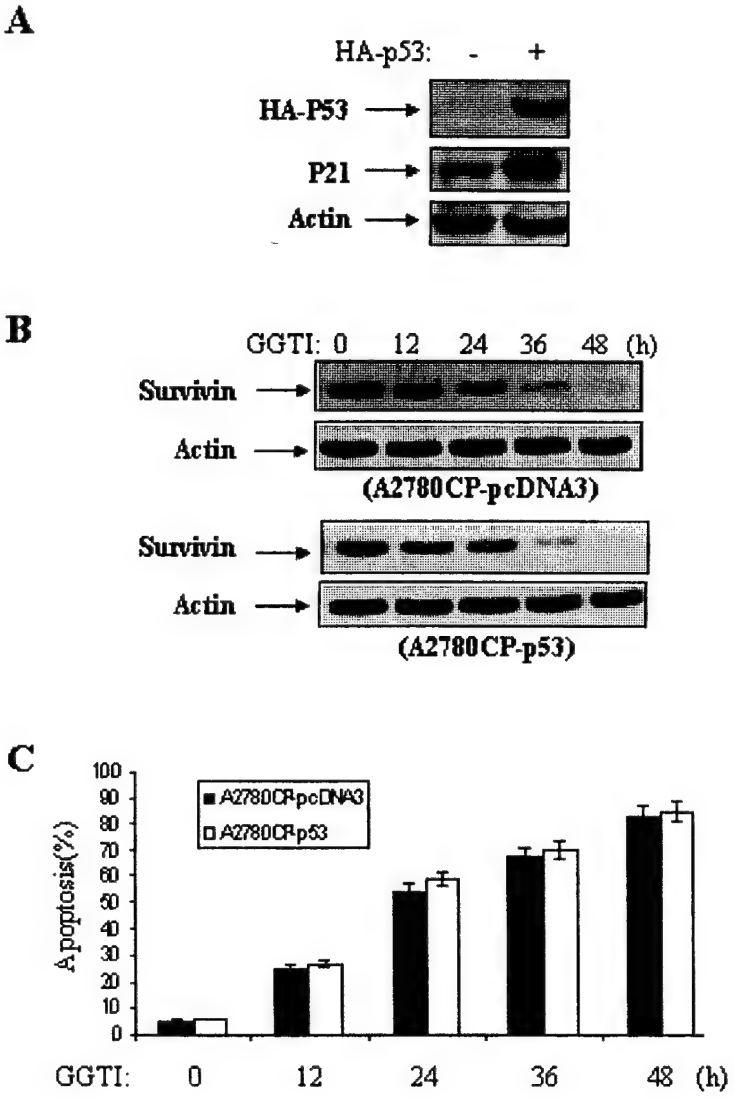

A
HA-p53: - +
HA-P53
P21
Actin
B
GGTI: 0 12 24 36 48 (h)
Survivin
Actin
(A2780CP-pcDNA3)
Survivin
Actin
(A2780CP-p53)
C
A2780CP-pcDNA3
A2780CP-p53
Apoptosis(%)
100
90
80
70
60
50
40
30
20
10
0
GGTI: 0 12 24 36 48 (h)

Fig. 5

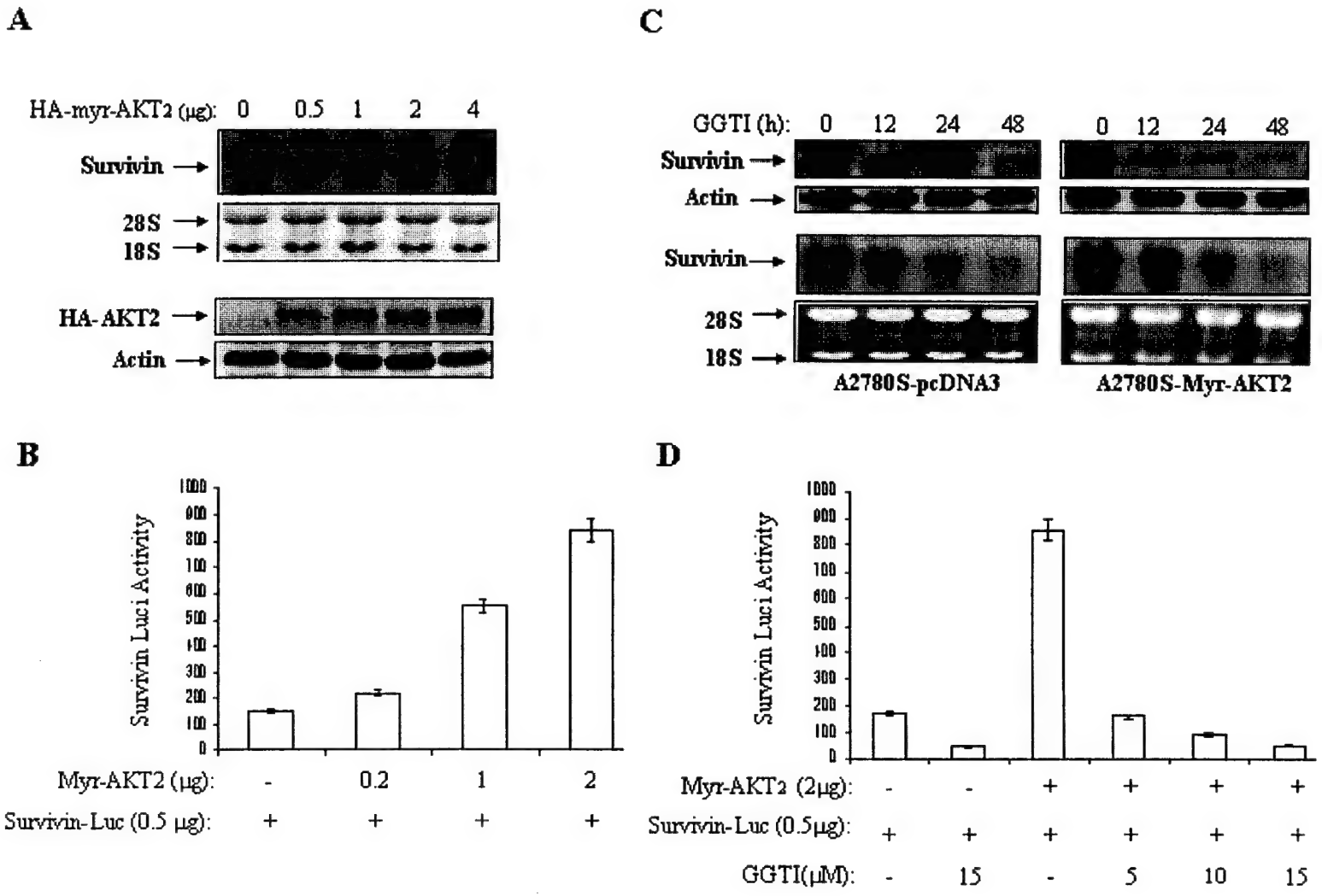

A
HA-myr-AKT2 (μg): 0 0.5 1 2 4
Survivin
28S
18S
HA-AKT2
Actin
C
GGTI (h): 0 12 24 48 0 12 24 48
Survivin
Actin
Survivin
28S
18S
A2780S-pcDNA3
A2780S-Myr-AKT2
B
Survivin Luci Activity
Myr-AKT2 (μg): - 0.2 1 2
Survivin-Luc (0.5 μg): + + + +
D
Survivin Luci Activity
Myr-AKT2 (2μg): - - + + + +
Survivin-Luc (0.5μg): + + + + + +
GGTI(μM): - 15 - 5 10 15

Fig. 6

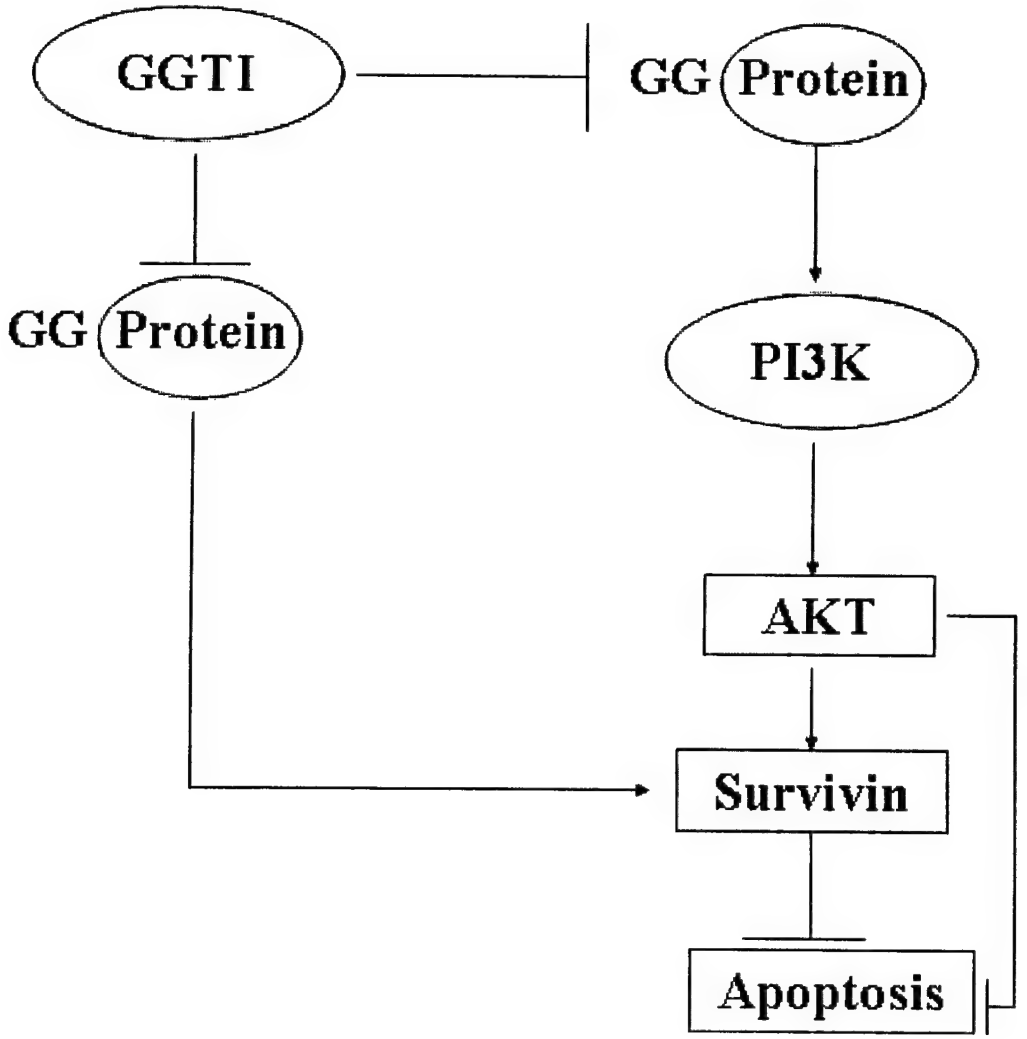


Fig. 7

Activation and Overexpression of Centrosome Kinase BTAK/Aurora-A in Human Ovarian Cancer[1]

Tatiana M. Gritsko,[2] Domenico Coppola,[2] June E. Paciga,[2] Lin Yang, Mei Sun, Sue A. Shelley, James V. Fiorica, Santo V. Nicosia, and Jin Q. Cheng[3]

Department of Pathology and Molecular Oncology Program, H. Lee Moffitt Cancer Center and Research Institute, College of Medicine, University of South Florida, Tampa, Florida 33612

ABSTRACT

Previous studies have demonstrated amplification of the centrosome serine/threonine kinase *BTAK/Aurora-A* in 10–25% of ovarian cancers. However, alterations of BTAK/Aurora-A at kinase and protein levels and its role in ovarian cancer progression have not been well documented. In this study, we examined the kinase activity and protein levels of BTAK/Aurora-A in 92 patients with primary ovarian tumors. *In vitro* kinase analyses revealed elevated BTAK/Aurora-A kinase activity in 44 cases (48%). Increased BTAK/Aurora-A protein levels were detected in 52 (57%) specimens. High protein levels of BTAK/Aurora-A correlated well with elevated kinase activity. Activation and overexpression of BTAK/Aurora-A were more frequently detected in early stage/low-grade ovarian tumors, although there was no statistic significance at the kinase level between early stage/low-grade and late stage/high-grade tumors. Moreover, BTAK/Aurora-A was preferentially expressed in noninvasive tumors, as revealed by immunohistochemical staining, suggesting that alterations of BTAK/Aurora-A could be an early event in human ovarian oncogenesis. To our knowledge, this is the first demonstration of recurrent activation and overexpression of BTAK/Aurora-A in human ovarian cancer, which may play a critical role in development of this malignancy.

INTRODUCTION

BTAK/Aurora-A (also named STK15, aurora-2, ARKI, and AIKI) is a serine/threonine protein kinase that belongs to the *Drosophila* aurora and *Saccharomyces cerevisiae* Ipl1 (Aurora/Ipl1p) kinase family and is essential for chromosome segregation and centrosome functions (1–3). In proliferating cells, expression of BTAK/Aurora-A is regulated in a cell cycle-dependent manner; its protein level is low in G_1-S, up-regulated during G_2-M, and reduced rapidly after mitosis (4). Immunofluorescence analysis revealed that BTAK/Aurora-A is localized to the spindle pole during mitosis, especially from prophase through anaphase (3, 4). Moreover, it has been shown that BTAK/Aurora-A interacts with Cdc20 and protein phosphatase 1 and induces cyclin B translation by phosphorylation of CPEB[4] (3) to regulate mitotic cell division (5–7). These studies suggest that BTAK/Aurora-A plays a critical role in regulation of centrosome function(s), and, thus, its alterations could result in chromosomal instability and malignant transformation. In fact, ectopic expression of BTAK/Aurora-A in Rat1 and NIH3T3 cells induces centrosome amplification, aneuploidy, and oncogenic phenotype (2, 3).

Recent studies have shown that the molecular mechanism of BTAK/Aurora-A regulation of G_2-M transition is because of phosphorylation of histone H3 (8, 9), a key molecule in conversion of the relaxed interphase chromatin to mitotic condensed chromosomes, a process likely to be essential for the subsequent nuclear division (10). Histone H3 is phosphorylated during mitosis on at least two serine residues, Ser-10 (11, 12) and Ser-28 (13, 14). Phosphorylation at Ser-10 in the histone H3 tail, which occurs early in the G_2 phase within pericentromeric heterochromatin and which by metaphase has spread throughout all chromosomal region, is considered to be a crucial event for the onset of mitosis. Phosphorylation on Ser-28 only becomes evident in early mitosis. It has been demonstrated in yeast, nematodes, and mammalian cells that BTAK/Aurora-A physically interacts with histone H3 and phosphorylates both Ser-10 and Ser-28 (8, 9). In addition, a recent report shows that BTAK/Aurora-A phosphorylates CPEB on Ser-174, which is necessary for cyclin B1 RNA polyadenylation-induced translation and entry into M phase (7). These data indicate that the Aurora kinase family plays a pivotal role during the G_2-M transition.

The *BTAK/Aurora-A* gene was mapped to human chromosome 20q13.2–13.3, a region frequently shown to be amplified in human carcinomas of breast, ovary, and colon (2). In fact, previous studies showed that the *BTAK/Aurora-A* was amplified in 15–25% of ovarian cancer cell lines and primary tumors (3, 15). In the present study, we show elevated kinase and protein levels of BTAK/Aurora-A in about half of the primary ovarian cancer specimens examined, indicating that alterations of

Received 9/13/02; revised 12/9/02; accepted 12/10/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by Grants CA89242 and CA77935 from NIH and DAMD17-00-0559 and DAMD17-02-1-0671 from the Department of Defense.

[2] T. M. G., D. C., and J. E. P. contributed equally to this work.

[3] To whom requests for reprints should be addressed, at Department of Pathology and Molecular Oncology program, H. Lee Moffitt Cancer Center, University of South Florida College of Medicine, 12901 Bruce B. Downs Boulevard, MDC Box 11, Tampa, FL 33612. Phone: (813) 974-8595; Fax: (813) 974-5536; E-mail: jcheng@hsc.usf.edu.

[4] The abbreviations used are: CPEB, cytoplasmic polyadenylation element-binding protein; MBP, myelin basic protein; GST, glutathione *S*-transferase; HEK, human embryonic kidney; LMP, carcinomas of low malignant potential; HA, hemagglutinin.

Table 1 Alterations of BTAK/Aurora-A and tumor histopathology

Histology	*n*	BTAK protein level		BTAK kinase activity	
		Low/no	High/moderate	Low	High
LMP	10	2	8	2	8
Serous cystadenocarcinoma	58	27	31	31	27
Mucinous cystadenocar.	12	4	8	7	5
Endometrioid cystadenocar.	6	2	4	3	3
Clear cell cystadenocar.	1	1	0	1	0
Granulosa cell tumor	4	4	0	4	0
Mixed tumor	1	0	1	0	1

BTAK/Aurora-A at the kinase and protein levels are common events, which could play a pivotal role in human ovarian oncogenesis.

MATERIALS AND METHODS

Tumor Specimens, Cell Lines, Transfection, and Statistic Analysis. All of the primary human ovarian cancer specimens were obtained from patients who underwent surgery at H. Lee Moffitt Cancer Center, and each sample contained ≥80% tumor cells, as was confirmed by microscopic examination. The tissues were snap frozen and stored at −70°C. Histopathologically, the ovarian cancer specimens include 10 LMP, 58 serous, 12 mucinous, and 6 endometroid ovarian surface epithelial cystadenocarcinomas, 1 clear cell carcinoma, and 1 mix tumor. We also evaluated 4 granulosa cell tumors (Table 1). Six normal tissues adjacent to tumors and normal ovaries were used as controls. Slides from each case were reviewed for grade following the criteria of the American Joint Committee on Cancer, 1988 edition. HEK293 cells were cultured at 37°C in DMEM supplemented with 10% FCS. Transfection was carried out with calcium phosphate. The relationship between the alteration of BTAK/Aurora-A and tumor grade and stage was analyzed with χ^2 tests.

Expression Constructs, GST Fusion Protein, and Production of Anti-BTAK/Aurora-A Antibody. The pcDNA3-*BTAK/Aurora-A* was kindly provided by Dr. Subrata Sen (The University of Texas M. D. Anderson Cancer Center). We subcloned HA epitope-tagged, wild-type *BTAK/Aurora-A* (1.2 kb) at the *Not*I sites of the mammalian expression vector pHM6 (Boehringer Mannheim). The GST-BTAK/Aurora-A was created by PCR amplification of aurora box-2 of BTAK/Aurora-A (2) using primers 5′-CAGGCTCAGCGGGTCTTGTGTC-3′ and 5′-CAGTTCCTCCTCAGGATT-3′. The PCR products were inserted into pGEX-4T vector. Logarithmically growing cultures of *Escherichia coli* TOP10 transformed with the pGEX-4T recombinant was incubated with 0.1 mM isopropyl-D-thiogalactopyranoside at 37°C for 6 h. The cells were pelleted, resuspended in cold PBS, and sonicated on ice. Debris was removed by centrifugation, and the supernatant was applied to a glutathione-sepharose 4B column (Pharmacia Biotech). GST-BTAK/Aurora-A fusion protein was eluted. Anti-BTAK/Aurora-A antibodies were raised in New Zealand White rabbits. Approximately 300 μg of GST fusion protein were used to immunize each rabbit every 2 weeks; rabbits were bled 12 days after each booster injection. The antibodies were affinity purified.

Immunoprecipitation and Western Blotting Analyses. The frozen tissue was lysed by a Tissue Tearor in a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1% NP40, 5 mM EGTA (pH 7.5), 1 mM EDTA (pH 8.0), 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin and leupeptin, 2 mM benzamidine, 10 mM NaF, 10 mM $NaPP_i$, 1 mM sodium vanadate, and 25 mM β-glycerolphosphate. Lysates were centrifuged at 12,000 × *g* for 15 min at 4°C. Equal amounts of protein lysate were analyzed for BTAK/Aurora-A expression and enzyme activity. For immunoprecipitation, lysates were precleared with protein A-protein G (2:1) agarose beads at 4°C for 20 min. After removal of the beads by centrifugation, lysates were incubated with anti-BTAK/Aurora-A polyclonal antibody in the presence of 30 μl of protein A:protein G (2:1) agarose beads (Life Technologies, Inc.) for 2 h at 4°C. The beads were washed three times with the lysis buffer. Protein expression was determined by Western blotting analyses probed with anti-BTAK/Aurora-A or anti-HA antibody. Detection of antigen bound antibody was carried out with the ECL Western Blotting Analysis System (Amersham).

***In Vitro* Protein Kinase Assay.** The immunoprecipitation for BTAK/Aurora-A kinase assay was performed as described above. The beads were washed three times with lysis buffer and two times with kinase buffer [100 mM Tris-HCl (pH 7.5), 2 mM EDTA (pH 8), 20 mM $MgCl_2$,10 mM $MnCl_2$, and 1 mM DTT] in the presence of the protease inhibitors. The reaction was carried out with 10 μCi of [^{32}P]ATP and 3 μM unlabeled ATP in 30 μl of kinase buffer. MBP (4 μg) was used as exogenous substrate. After incubation at 37°C for 30 min, the reaction was stopped by adding protein-loading buffer and separated by SDS-PAGE. Each experiment was repeated three times. The relative amounts of incorporated radioactivity were determined by autoradiography and quantified with a PhosphorImager (Molecular Dynamics).

Southern and Northern Blotting Analyses. Genomic DNA and total RNA were isolated from the human ovarian tumor specimens by standard methods (2). Southern blots were prepared by digestion of 10 μg of DNA with EcoRI and detected with a random primer [^{32}P]dCTP-labeled *BTAK/Aurora-A* cDNA probe. Autoradiographs were quantified relative to β-actin using Image-Quant software. For Northern blotting analyses, 20 μg of total RNA were electrophoresed on agarose gel, transferred, and detected with *BTAK/Aurora-A* cDNA probe.

Immunohistochemistry. Formalin-fixed, paraffin-embedded sections were subjected to antigen retrieval by boiling in 0.01 M sodium citrate buffer (pH 6.0) in a microwave oven after

dewaxing and rehydration. The Vectastain ABC Kit for rabbit IgG (Vector Laboratories) was used to immunostain the tissue sections with anti-BTAK/Aurora-A antibody. Endogenous peroxidase and biotin were blocked, and sections were incubated 1 h at room temperature with a 1:250 dilution of antibody to BTAK/Aurora-A. The remainder of the staining procedure was performed according to the manufacturer's instructions using diaminobenzidine tetrahydrochloride as the chromogen and hematoxylin for counterstaining. Primary antibody was replaced with an equal concentration of nonimmune rabbit IgG on control sections.

RESULTS

Characterization of BTAK/Aurora-A Antibody. Previous studies have demonstrated amplification of the *BTAK/Aurora-A* in 10–25% of human ovarian cancer (3, 15). However, alterations of BTAK/Aurora-A at kinase and protein levels have not been documented. To examine BTAK/Aurora-A kinase activity and protein expression in human ovarian carcinoma, we generated rabbit polyclonal anti-BTAK/Aurora-A antibody. The specificity of the antibody was examined with HEK293 cells transfected with the HA-*BTAK/Aurora-A* expression plasmid. Western blotting analyses revealed that anti-BTAK/Aurora-A antibody reacted strongly with BTAK/Aurora-A protein (Fig. 1*A*). After preincubation of anti-BTAK/Aurora-A antibody with GST-BTAK/Aurora-A antigen, no BTAK protein was detected (Fig. 1*B*). In addition, we have further examined the usefulness of the anti-BTAK/Aurora-A antibody for immunoprecipitation. Immunoprecipitates were prepared with anti-BTAK/Aurora-A antibody in HEK293 cells transfected with the HA-*BTAK/Aurora-A* expression construct, separated in SDS-PAGE, and detected with anti-HA antibody. As shown in Fig. 1*C*, anti-BTAK/Aurora-A antibody is capable of precipitating BTAK/Aurora-A protein from the cell lysate. These results indicate that our anti-BTAK/Aurora-A antibody specifically reacts with BTAK/Aurora-A and works for both Western blot and immunoprecipitation.

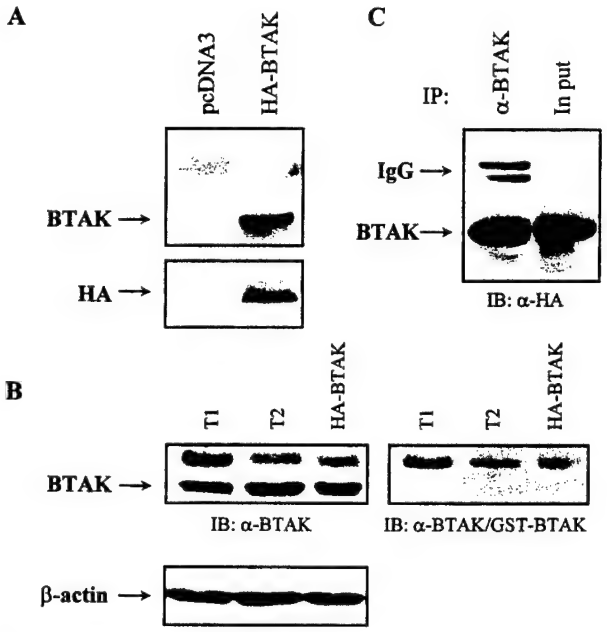


Fig. 1 Characterization of anti-BTAK/Aurora-A antibody. In *A*, BTAK/Aurora-A antibody reacts with high levels of BTAK/Aurora-A protein. HEK293 cells were transfected with pcDNA3 or pcDNA3-HA-tagged *BTAK/Aurora-A* expression plasmid. Cell lysates were subjected to Western blotting analyses with anti-BTAK/Aurora-A (*top*) or anti-HA (*bottom*) antibody. Endogenous BTAK/Aurora-A in HEK293 cells is undetectable (*left lane* of *top panel*). In *B*, anti-BTAK/Aurora-A antibody specifically reacts with BTAK/Aurora-A. Two tumor specimens overexpressing BTAK/Aurora-A- and HA-*BTAK/Aurora-A*-transfected HEK293 cell lysates were detected with anti-BTAK/Aurora-A antibody (*top left panel*). The same blot was probed with anti-BTAK/Aurora-A serum preincubated with GST-BTAK/Aurora-A antigen (*top right panel*) or β-actin (*bottom*) antibody. In *C*, the BTAK/Aurora-A antibody is able to immunoprecipitate BTAK/Aaurora-A protein. HA-*BTAK/Aurora-A*-transfected HEK293 cells were lysed and incubated with anti-BTAK/Aurora-A antibody in the presence of protein A:G. The immunoprecipitates were subjected to immunoblotting analyses with anti-HA antibody.

Frequent Activation of BTAK/Aurora-A Kinase in Primary Ovarian Tumors. As BTAK/Aurora-A is a serine/threonine kinase and plays a significant role in cell proliferation by phosphorylation of downstream targets, such as histone H3 and CPEB (7–9), we have examined BTAK/Aurora-A kinase activity in human primary ovarian carcinoma. *In vitro* BTAK/Aurora-A kinase assays were performed in 92 specimens using MBP as substrate. Fig. 2*A* shows that the *in vitro* kinase conditions that we used could detect high levels of BTAK/Aurora-A kinase activity in *BTAK/Aurora-A*- but not pcDNA3-transfected HEK293 cells. Activation of BTAK/Aurora-A, defined as an average reading of the kinase activity 4-fold higher than that in normal ovarian tissues, was detected in 44 (48%) ovarian cancer specimens. The results were confirmed by triplicate experiments. Fig. 2*B* represents typical examples of BTAK/Aurora-A activation in ovarian tumors.

In addition, we have examined the relationship between kinase activity and protein level of BTAK/Aurora-A. Western blot and immunohistochemical staining analyses revealed that all of the 44 cases with elevated levels of BTAK/Aurora-A kinase overexpressed BTAK/Aurora-A, indicating that activation of BTAK/Aurora-A is largely attributable to increased expression levels of BTAK/Aurora-A protein. To determine whether elevated protein levels of BTAK/Aurora-A result from its alterations at DNA and/or mRNA levels, we performed Northern and Southern blotting analyses in 43 tumor specimens and 6 normal ovarian tissues. Overexpression of *BTAK/Aurora-A* mRNA was detected in 18 (42%) tumors, in which BTAK/Aurora-A protein was elevated, whereas amplification of the *BTAK/Aurora-A* (>3-fold higher than that in normal ovary) was only observed in 6 (14%) specimens, all of which also exhibited overexpression of the *BTAK/Aurora-A* mRNA and protein. Representative examples are shown in Fig. 3, *B* and *C*, suggesting that BTAK/Aurora-A could be regulated at transcription, translation, and/or post-translational levels.

Elevated Expression of BTAK/Aurora-A Protein in Human Ovarian Carcinomas. We next examined the expression of BTAK/Aurora-A protein in the same series of human ovarian tumors. Western blotting analyses revealed high levels of BTAK/Aurora-A protein in 52 of 92 (57%) tumor specimens (Fig. 3*A* and Table 1). To confirm this result, we have carried out immunohistochemical staining of paraffin sections with anti-BTAK/Aurora-A

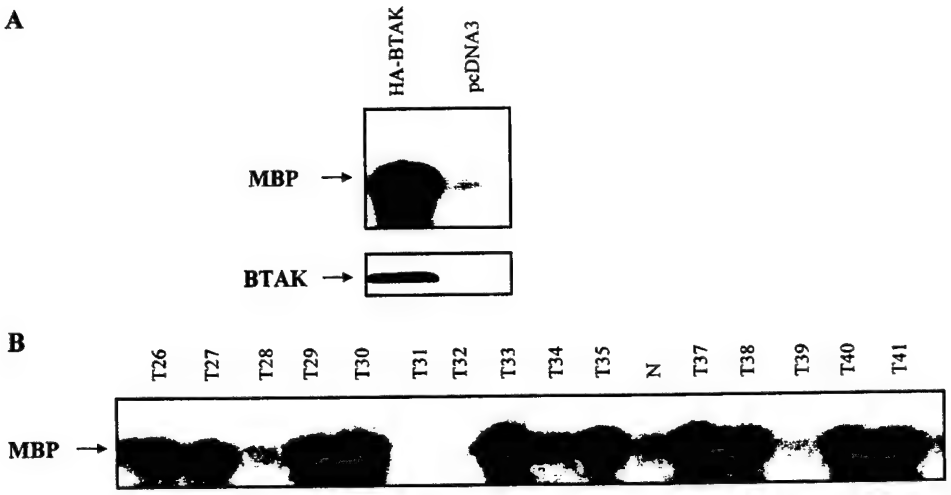


Fig. 2 Activation of BTAK/Aurora-A in human ovarian cancer. *A, in vitro* kinase assay analysis of BTAK/Aurora-A immunoprecipitates prepared from *BTAK/Aurora-A*- (*left*) and pcDNA3 (*right*) -transfected HEK293 cells with anti-BTAK/Aurora-A antibody. MBP was used as substrate (*top*). *Bottom panel*, Western blot probed with anti-BTAK/Aurora-A antibody. *B, in vitro* kinase assays of BTAK/Aurora-A immunoprecipitates from 15 representative frozen ovarian tumor specimens and a normal ovarian tissue (*N*). Tissue lysates were incubated with anti-BTAK/Aurora-A antibody in the presence of protein A-protein G agarose beads for 2 h. After extensive washes, immunoprecipitates were subjected to *in vitro* kinase assay using MBP as the exogenous substrate. Each experiment was repeated three times. The relative amounts of incorporated radioactivity were determined by autoradiography and quantitated with a PhosphorImager.

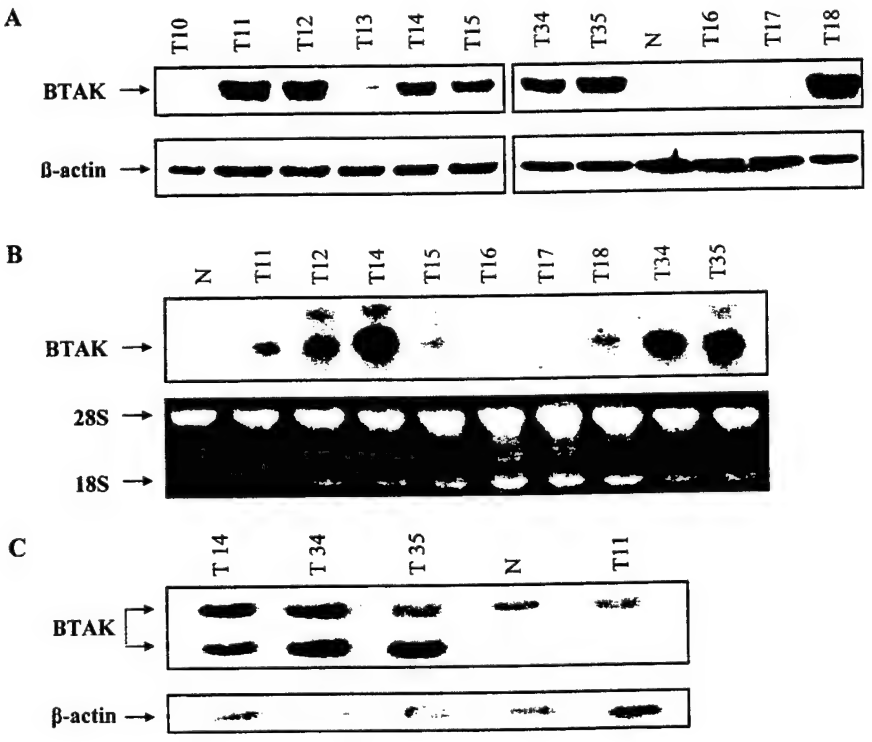


Fig. 3 Overexpression of BTAK/Aurora-A in ovarian carcinoma. *A*, Western blotting analyses of human primary ovarian tumor specimens (*T*) and normal ovarian tissue (*N*). Sixty μg of protein from each specimen were separated in SDS-PAGE, transferred, and detected with anti-BTAK/Aurora-A antibody (*top*). A weak low molecular weight band detected in N, T16, and T17 could be an alternatively spliced form because preincubation of BTAK/Aurora-A antibody with GST-BTAK/Aurora-A antigen is able to compete this band (data not shown). Equal loadings were indicated by hybridization of the same blot with anti-β-actin antibody (*bottom*). *B*, Northern blotting analyses of expression of *BTAK/Aurora-A* mRNA with [^{32}P]-dCTP-labeled *BTAK/Aurora-A* cDNA as probe (*top*). The quality and loading amount of RNA were indicated by 28S and 18S ribosome RNA (*bottom*). In *C*, Southern blots containing 10 μg of *Pst*I-digested DNA per lane from primary human ovarian tumors and a normal ovary control were hybridized with a *BTAK/Aurora-A* cDNA probe. The location of the 2.8 and 2.5 kb of *BTAK/Aurora-A* fragments is marked. Blot was stripped and reprobed with a human β-actin probe to confirm equal loading.

antibody. A moderate to strong predominantly cytoplasmic BTAK/Aurora-A expression was detected in the same 52 ovarian tumors (Fig. 4) that overexpress BTAK/Aurora-A protein revealed by Western blotting analyses. It has been shown that although BTAK/Aurora-A protein localizes to both the cytoplasm and nucleus, it is mainly in cytoplasm (16). Strong immunoreaction of BTAK/Aurora-A was observed in tumor cells but not normal ovarian epithelium. There was no preferential BTAK/Aurora-A expression among the three major histopathological types of ovarian surface epithelial carcinomas (serous, mucinous, and endometroid). Four

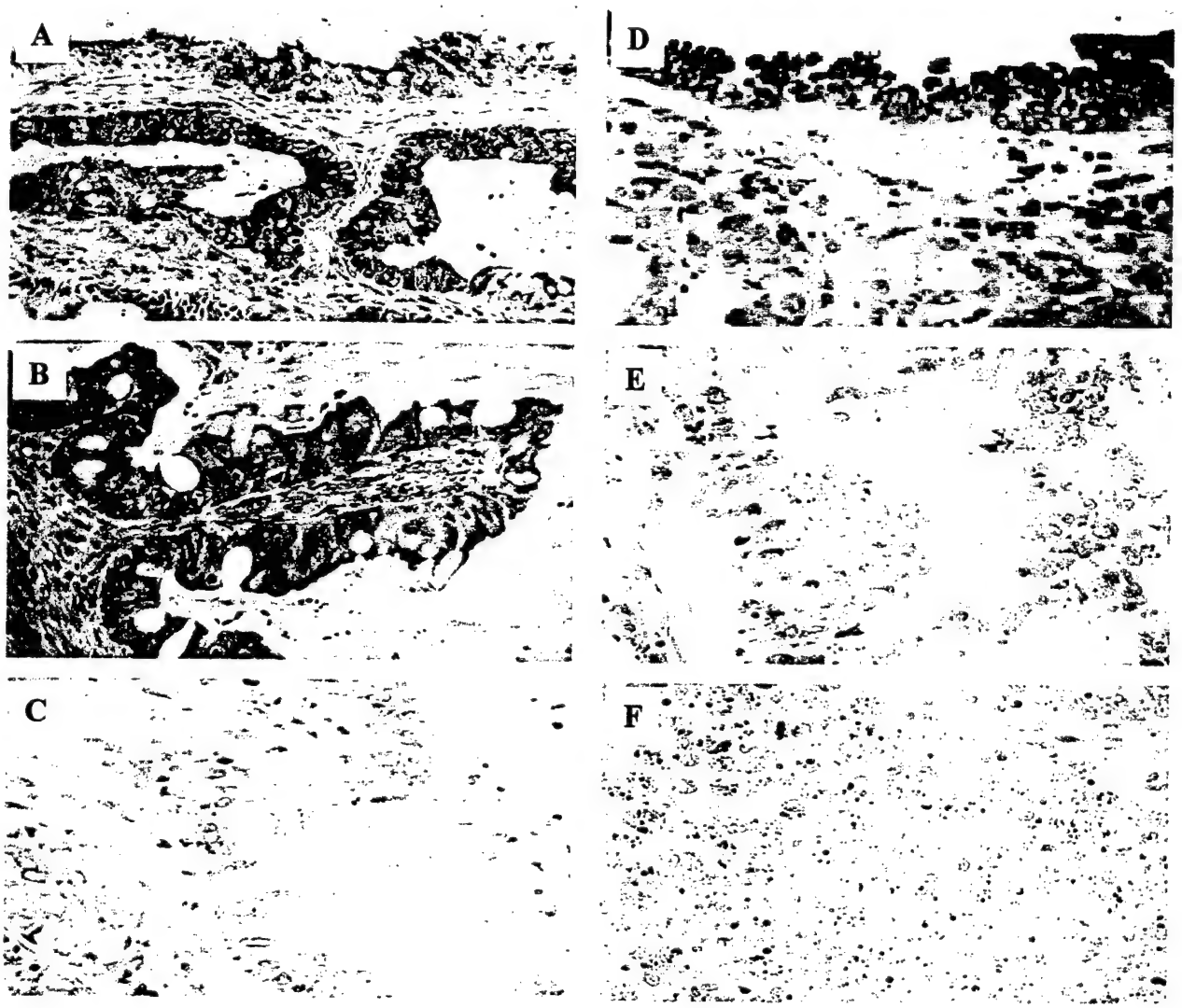


Fig. 4 High levels of BTAK/Aurora-A protein expression in low-grade and less invasive tumors. Immunohistochemical staining of the paraffin sections prepared from mucinous papillary tumors of low malignant potential (*A* and *B*), invasive mucinous adenocarcinoma (*C*), and serous adenocarcinoma (*D–F*) with anti-BTAK/Aurora-A antibody. The intensity of the immunostaining in invasive tumors is much weaker or absent in comparison with noninvasive tumors.

ovarian granulosa cell tumors exhibited no detectable BTAK/Aurora-A, implying that the expression of BTAK/Aurora-A could be restricted to ovarian epithelial neoplasm. Notably, activation/overexpression of BTAK/Aurora-A is more frequently detected in LMP (8 of 10) than serous and mucinous cystadenocarcinoma (39 of 70). Furthermore, we have observed that BTAK/Aurora-A is preferentially expressed in low-grade [25 of 39 (65%) grade I/II *versus* 13 of 33 (40%) grade III] and early stage tumors [24 of 35 (67%) stage I/II *versus* 9 of 20 (45%) stage III/IV] (Tables 2 and 3). However, there is no preference in six cases with amplification of BTAK/Aurora-A (one case is stage I, two cases are stage II, and the rest are stage III). In addition, invasive tumors exhibit much less BTAK/Aurora-A immunoreactivity compared with the noninvasive tumors (Fig. 4). Interestingly, even in the same tumor, BTAK/Aurora-A immunoreactivity was seen to pale at the invasive front of the tumor, whereas the noninvasive portion of the tumor stained strongly. Fig. 4*D* shows an invasive serous adenocarcinoma exhibiting BTAK/Aurora-A expression. However, the intensity of the staining was much less in the infiltrating component compared with the LMP component lining the surface. These data suggest that alterations of BTAK/Aurora-A could be an early event in the development of human ovarian cancer, al though there was no statistic significance at the kinase level between early stage/low-grade and late stage/high-grade tumors (Tables 2 and 3).

DISCUSSION

Ovarian cancer is thought to arise from alterations in genes involved in regulating cell proliferation, apoptosis, and genomic integrity. Alterations in several proto-oncogenes and tumor sup-

Table 2 Protein and kinase levels of BTAK/Aurora-A and tumor grade

		BTAK protein level			BTAK kinase activity		
Grade	*n*	Low/no	High/moderate	*P*	High	Low	*P*
I-II	39	14	25	<0.05	20	19	>0.05
III	33	20	13		21	12	

Table 3 Protein and kinase levels of BTAK and clinical stage

		BTAK protein level			BTAK kinase activity		
Stage	*n*	Low/no	High/moderate	*P*	High	Low	*P*
I-II	35	11	24	<0.05	15	20	>0.05
III-IV	49	27	22		30	19	

pressor genes have been described. The *ERBB2*, *PIK3CA*, and *AKT2* oncogenes are frequently amplified and subsequently overexpressed in ovarian cancers (17–19). Overexpression of ERBB2 or AKT2 correlates with poor prognosis of the patients (17, 18). Amplification of the *MYC* oncogene has been detected in ~20% of ovarian cancers, more frequently in serous than in mucinous cancers (20). Other oncogenes altered in ovarian cancer include *KRAS*, *INT2*, *FMS*, and *MDM2*, but these alterations appear to be relatively uncommon (17). Cytogenetic and comparative genome hybridization studies have revealed frequent gains in chromosome 20q11–13 copy number in ovarian cancer (21). Several putative candidate oncogenes from this region have recently been identified, including *AIB3* and *AIB4* mapping to 20q11, *AIB1* gene at 20q12, *MYBL2* and phosphotyrosine-phosphatase 1 genes at 20q13, and *ZNF217* and *BTAK/Aurora-A* genes at 20q13.2 amplicon (3, 15, 22). Amplification of the *BTAK/Aurora-A* has been reported in 15–25% ovarian cancer cell lines and primary tumors (3, 15). In the present study, we have studied kinase activity and protein expression of BTAK/Aurora-A in primary ovarian carcinomas. Elevated kinase activity and protein levels of BTAK/Aurora-A were detected in 48 and 57% of the tumors examined, respectively. These data indicate that alterations of BTAK/Aurora-A at kinase and protein levels are frequent changes in human ovarian cancer. Thus, BTAK/Aurora-A could play a pivotal role in the pathogenesis in the majority of cases of this malignancy.

We have also examined DNA amplification and mRNA overexpression of the *BTAK/Aurora-A* in 43 tumors. Frequency of alterations of the *BTAK/Aurora-A* at DNA level is much lower than at protein and kinase levels. Moreover, overexpression of *BTAK/Aurora-A* mRNA is much more common than amplification of *BTAK/Aurora-A*. A possible reason for these findings is insensitive detection of amplification by Southern blot analyses. Interphase fluorescent *in situ* hybridization has been reported to be more sensitive for detection of gene amplification. A recent report using fluorescent *in situ* hybridization demonstrated amplification of *BTAK/Aurora-A* in 6 of 24 (25%) sporadic ovarian carcinomas (15). A second possibility to explain the findings relates to the transcriptional regulation of BTAK/Aurora-A. In addition, our data show that overexpression of BTAK/Aurora-A protein is ~20% higher than overexpression of its mRNA in the human primary ovarian tumors examined, which could be because of RNA quality. However, based on 28S and 18S bands in our Northern blots, RNA degradation did not occur in any of the 43 tumors we examined (Fig. 3), suggesting that translational and/or post-translational regulation could be involved in up-regulation of BTAK/Aurora-A protein in human ovarian carcinoma.

We have also observed that BTAK/Aurora-A kinase activity correlates well with its protein expression in ovarian tumor specimens examined, except in 8 cases exhibiting increased protein but not kinase levels of BTAK/Aurora-A. We and others (23, 24) have shown previously that Akt protein kinase and Stat3 DNA-binding activities in primary tumors are closely associated with the interval time to freezing the specimen after surgical resection. The 8 ovarian tumors, which have elevated levels of BTAK/Aurora-A protein but not BTAK/Aurora-A kinase, also overexpress AKT2 protein but have low levels of AKT2 kinase activity (18), indicating that these results could be attributable to improper processing and/or storage of the specimens. Nevertheless, the majority of tumors with overexpressed BTAK/Aurora-A protein displayed increased levels of BTAK/Aurora-A kinase activity, suggesting that the activation of BTAK/Aurora-A is largely caused by overexpression of its protein in the primary ovarian tumors examined in this study.

The relationship between overexpression of BTAK/Aurora-A and tumor grade/stage is controversial. A previous study in ductal breast cancer showed that overexpression of BTAK/Aurora-A protein was independent of tumor histopathological type and lacked correlation with tumor size and lymph node metastases (16). Other studies showed that alterations of BTAK/Aurora-A associate with poor prognosis in gastric cancers and high grade/late stage in breast and bladder cancer (25–27). In the present study, however, we observed that BTAK/Aurora-A protein kinase is preferentially activated/overexpressed in low-grade and early stage ovarian cancer, as well as LMP (Tables 1–3), although there was no statistic significance at the kinase level between low-grade/early stage and high-grade/late stage tumors. Moreover, immunohistochemical staining showed that BTAK/Aurora-A is preferentially expressed in less invasive tumors and declines once a tumor becomes invasive (Fig. 4, *D–F*). A recent report using a well-established rat mammary cancer model demonstrated that amplification of the *BTAK/Aurora-A* is an early genetic change during the development of

rat mammary carcinoma (28). We have also documented that ectopic expression of BTAK/Aurora-A significantly induces telomerase activity, which is required for cell immortalization and transformation.[5] Therefore, activation and overexpression of BTAK/Aurora-A protein kinase may represent early changes and play an important role in development of a subset of human ovarian cancers.

ACKNOWLEDGMENTS

We thank the Tissue Procurement and Molecular Biology Core Facilities at H. Lee Moffitt Cancer Center and Research Institute for providing tumor specimens and sequencing.

REFERENCES

1. Glover, D. M., Leibowitz, M. H., McLean, D. A., and Parry, H. Mutations in aurora prevent centrosome separation leading to the formation of monopolar spindles. Cell, *81:* 95–105, 1995.

2. Bischoff, J. R., Anderson, L., Zhu, Y., Mossie, K., Ng, L., Souza, B., Schryver, B., Flanagan, P., Clairvoyant, F., Ginther, C., Chan, C. S., Novotny, M., Slamon, D. J., and Plowman, G. D. A homologue of Drosophila aurora kinase is oncogenic and amplified in human colorectal cancers. EMBO J., *17:* 3052–3065, 1998.

3. Zhou, H., Kuang, J., Zhong, L., Kuo, W. L., Gray, J. W., Sahin, A., Brinkley, B. R., and Sen, S. Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation. Nat. Genet., *20:* 189–193, 1998.

4. Kimura, M., Kotani, S., Hattori, T., Sumi, N., Yoshioka, T., Todokoro, K., and Okano, Y. Cell cycle-dependent expression and spindle pole localization of a novel human protein kinase, Aik, related to Aurora of Drosophila and yeast Ipl1. J. Biol. Chem., *272:* 13766–13771, 1997.

5. Farruggio, D. C., Townsley, F. M., and Ruderman, J. V. Cdc20 associates with the kinase aurora2/Aik. Proc. Natl. Acad. Sci. USA, *96:* 7306–7311, 1999.

6. Katayama, H., Zhou, H., Li, Q., Tatsuka, M., and Sen, S. Interaction and feedback regulation between STK15/BTAK/Aurora-A kinase and protein phosphatase 1 through mitotic cell division cycle. J. Biol. Chem., *276:* 46219–46224, 2001.

7. Groisman, I., Jung, M. Y., Sarkissian, M., Cao, Q., and Richter, J. D. Translational control of the embryonic cell cycle. Cell, *109:* 473–483, 2002.

8. Crosio, C., Fimia, G. M., Loury, R., Kimura, M., Okano, Y., Zhou, H., Sen, S., Allis, C. D., and Sassone-Corsi, P. Mitotic phosphorylation of histone H3: spatio-temporal regulation by mammalian Aurora kinases. Mol. Cell. Biol., *22:* 874–885, 2002.

9. Scrittori, L., Hans, F., Angelov, D., Charra, M., Prigent, C., and Dimitrov, S. pEg2 aurora-A kinase, histone H3 phosphorylation, and chromosome assembly in Xenopus egg extract. J. Biol. Chem., *276:* 30002–30010, 2001.

10. Hirano, T. Chromosome cohesion, condensation, and separation. Annu. Rev. Biochem., *69:* 115–144, 2000.

11. Paulson, J. R., and Taylor, S. S. Phosphorylation of histones 1 and 3 and nonhistone high mobility group 14 by an endogenous kinase in HeLa metaphase chromosomes. J. Biol. Chem., *257:* 6064–6072, 1982.

12. Wei, Y., Mizzen, C. A., Cook, R. G., Gorovsky, M. A., and Allis, C. D. Phosphorylation of histone H3 at serine 10 is correlated with chromosome condensation during mitosis and meiosis in Tetrahymena. Proc. Natl. Acad. Sci. USA, *95:* 7480–7484, 1998.

13. Goto, H., Tomono, Y., Ajiro, K., Kosako, H., Fujita, M., Sakurai, M., Okawa, K., Iwamatsu, A., Okigaki, T., Takahashi, T., and Inagaki, M. Identification of a novel phosphorylation site on histone H3 coupled with mitotic chromosome condensation. J. Biol. Chem., *274:* 25543–25549, 1999.

14. Goto, H., Yasui, Y., Nigg, E. A., and Inagaki, M. Aurora-B phosphorylates Histone H3 at serine28 with regard to the mitotic chromosome condensation. Genes Cells, *7:* 11–17, 2002.

15. Tanner, M. M., Grenman, S., Koul, A., Johannsson, O., Meltzer, P., Pejovic, T., Borg, A., and Isola, J. J. Frequent amplification of chromosomal region 20q12–q13 in ovarian cancer. Clin. Cancer Res., *6:* 1833–1839, 2000.

16. Tanaka, T., Kimura, M., Matsunaga, K., Fukada, D., Mori, H., and Okano, Y. Centrosomal kinase AIK1 is overexpressed in invasive ductal carcinoma of the breast. Cancer Res., *59:* 2041–2044, 1999.

17. Lynch, H. T., Casey, M. J., Lynch, J., White, T. E., and Godwin, A. K. Genetics and ovarian carcinoma. Semin. Oncol., *25:* 265–280, 1998.

18. Yuan, Z. Q., Sun, M., Feldman, R. I., Wang, G., Ma, X., Jiang, C., Coppola, D., Nicosia, S. V., and Cheng, J. Q. Frequent activation of AKT2 and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/Akt pathway in human ovarian cancer. Oncogene, *19:* 2324–2330, 2000.

19. Shayesteh, L., Lu, Y., Kuo, W. L., Baldocchi, R., Godfrey, T., Collins, C., Pinkel, D., Powell, B., Mills, G. B., and Gray, J. W. PIK3CA is implicated as an oncogene in ovarian cancer. Nat. Genet., *21:* 99–102, 1999.

20. Pejovic, T. Genetic changes in ovarian cancer. Ann. Med., *27:* 73–78, 1995.

21. Tirkkonen, M., Tanner, M., Karhu, R., Kallioniemi, A., Isola, J., and Kallioniemi, O. P. Molecular cytogenetics of primary breast cancer by CGH. Genes Chromosomes Cancer, *21:* 177–184, 1998.

22. Collins, C., Rommens, J. M., Kowbel, D., Godfrey, T., Tanner, M., Hwang, S. I., Polikoff, D., Nonet, G., Cochran, J., Myambo, K., Jay, K. E., Froula, J., Cloutier, T., Kuo, W. L., Yaswen, P., Dairkee, S., Giovanola, J., Hutchinson, G. B., Isola, J., Kallioniemi, O. P., Palazzolo, M., Martin, C., Ericsson, C., Pinkel, D., Albertson, D., Li, W. B., and Gray, J. W. Positional cloning of ZNF217 and NABC1: genes amplified at 20q13.2 and overexpressed in breast carcinoma. Proc. Natl. Acad. Sci. USA, *95:* 8703–8708, 1998.

23. Sun, M., Wang, G., Paciga, J. E., Feldman, R. I., Yuan, Z. Q., Ma, X. L., Shelley, S. A., Jove, R., Tsichlis, P. N., Nicosia, S. V., and Cheng, J. Q. AKT1/PKBalpha kinase is frequently elevated in human cancers and its constitutive activation is required for oncogenic transformation in NIH3T3 cells. Am. J. Pathol., *159:* 431–437, 2001.

24. Garcia, R., Yu, C. L., Hudnall, A., Catlett, R., Nelson, K. L., Smithgall, T., Fujita, D. J., Ethier, S. P., and Jove, R. Constitutive activation of Stat3 in fibroblasts transformed by diverse oncoproteins and in breast carcinoma cells. Cell Growth Differ., *8:* 1267–1276, 1997.

25. Sakakura, C., Hagiwara, A., Yasuoka, R., Fujita, Y., Nakanishi, M., Masuda, K., Shimomura, K., Nakamura, Y., Inazawa, J., Abe, T., and Yamagishi, H. Tumour-amplified kinase BTAK is amplified and overexpressed in gastric cancers with possible involvement in aneuploid formation. Br. J. Cancer, *84:* 824–831, 2001.

26. Miyoshi, Y., Iwao, K., Egawa, C., and Noguchi, S. Association of centrosomal kinase STK15/BTAK mRNA expression with chromosomal instability in human breast cancers. Int. J. Cancer, *92:* 370–373, 2001.

27. Sen, S., Zhou, H., Zhang, R. D., Yoon, D. S., Vakar-Lopez, F., Ito, S., Jiang, F., Johnston, D., Grossman, H. B., Ruifrok, A. C., Katz, R. L., Brinkley, W., and Czerniak, B. Amplification/overexpression of a mitotic kinase gene in human bladder cancer. J. Natl. Cancer Inst. (Bethesda), *94:* 1320–1329, 2002.

28. Goepfert, T. M., Adigun, Y. E., Zhong, L., Gay, J., Medina, D., and Brinkley, W. R. Centrosome amplification and overexpression of aurora A are early events in rat mammary carcinogenesis. Cancer Res., *62:* 4115–4122, 2002.

[5] H. Yang, C. C. Ou, P. Kruk, S. V. Nicosia, and J. Q. Cheng. Serine/threonine kinase BTAK induces telomerase activity by activating hTERT transcription in human ovarian and breast epithelial cells, submitted for publication.

Curriculum Vitae

Jin Q. Cheng

Education:

Beijing Capital Medical School	M.D., M.S.	Medicine	1986
Paris-XIII University, Paris, France	Ph.D.	Molecular and Cellular Biology	1994

Positions Held:

Beijing Thoracic Tumor Institute	(Attending Doctor, Surgeon)	1986-1988
Paris-XIII University, Paris, France	(Ph.D. student)	1988-1990
Fox Chase Cancer Center, Philadelphia & Paris-XIII University, Paris, France	(Ph.D. Student & Postdoctoral)	1990-1994
Fox Chase Cancer Center, Philadelphia	(Senior Research Associate)	1994-1996
Fox Chase Cancer Center, Philadelphia	(Staff Scientist)	1996-1997
University of South Florida College of Medicine, H. Lee Moffitt Cancer Center	(Assistant Professor)	1997-2001
University of South Florida College of Medicine, H. Lee Moffitt Cancer Center	(Associate Professor)	2001-Present

Research Grants and Awards (Total Costs):

Acive:

1. AKT2 oncogene and human oncogenesis
   NIH R01 CA77935 — 2003.6-2008.6
   Principal Investigator — $1,205,315.00

2. AKT1 oncogene in carcinogenesis
   NIH R01 CA089242 — 2001.6-2006.6
   Principal Investigator — $1,141,875.00

3. AKT survival pathways and FTI-induced apoptosis
   NIH R01 CA85709 — 2001.1-2006.1
   Co-Principal Investigator, 20% — $957,000.00

4. Insulin signaling pathways regulating PKCβ splicing
   NIH R01 DK54393 — 2001.6-2006.6
   Co-Principal Investigator, 10% — $1,450,000.00

5. AKT2 oncogene and its associated protein, APBP, in human ovarian cancer (PO1 grant) — 2002.9-2006.9
   Department of Defense DAMD17-02-1-0671 — $506,759

Leader of Project 1

6. PI 3-kinase/Akt pathway and human ovarian epithelial cancer
   Department of Defense DAMD17-00-1-0559 2000.9-2003.9
   Principal Investigator $434,634.00

7. AKT2 oncogene and phosphoinositide 3-kinase in breast cancer
   Department of Defense DAMD17-01-1-0394 2001.9-2004.9
   Principal Investigator $434,634.00

8. Regulation of telomerase activity in ovarian cancer
   Department of Defense DAMD17-00-1-0565 2000.9-2003.9
   Co-Principal Investigator, 5% $434,634.00

9. Berlex Bioscience Support (Renewable)
   Akt as a target for cancer intervention 1998.6-2004.6
   Principal Investigator $53,000.00

Pending:

Disruption of AKT survival pathway for cancer intervention
NIH
Principal Investigator

Role of AKT pathway in human prostate cancer
DOD
Principal Investigator

Hornors and Awards:

1. NIH Investigator First Award 1996

2. Special Fellow Award, The Leukemia Society of America 1993
   Positional cloning of the chromosome 9p21 tumor suppressor

3. Young Investigator Travel Award of the International Association
   for the Study of Lung Cancer (IASLC) 1994

4. Scholar from French Cancer Research Association (L'ARC France) 1988-1990

Grant Review Panels:

VA Merit Review Boards (2001-present).
Department of Defense, Ovarian and breast cancer study sections (2002)
Austria FWF (Austria Science Fund) Review Boards (2203)

Publications:

Yang L, Sun M, Yang H, Dan HC, Nicosia SV, **Cheng JQ,** Akt/PKB Inhibits HtrA2/Omi-stimulated Apoptosis through Direct Disruption of its Serine Protease Activity. *Submitted to Genes & Development.*

Yang H., Ou C, Feldman FI, Kruk PA, Nicosia SV, **Cheng JQ.** Aurora-A Upregulates Human Telomerase Reverse Transcriptase through Activation of c-Myc Transcription. *Submitted to EMBO.*

Leung BM, Fraser M, Yan X, Dan HC, **Cheng JQ,** Tsang BK. Akt and p53 Are Determinants of Xiap-Mediated Chemoresistance in Human Ovarian Cancer. *Submitted to Cancer Res.*

Johnson NC, Dan HC, **Cheng JQ,** Kruk PA. Loss of XIAP in BCRA1 185delAG human ovarian surface epithelial cells results in aberrant active casepase 3 ubiquitination. *Submitted to Mol. Cancer Res.*

Dan C, Sun M, Kaneko S, Feldman RI, Nicosia SV, Wang H-G, Tsang BK, **Cheng JQ.** Akt Phosphorylation and Stabilization of X-linked Inhibitor of Apoptosis, XIAP: A Mechanism of Akt in Cell survival and Chemoresistance. *Submitted to JBC*

Sun M, Yang L, Feldman RI, Sun X, Jove R, Nicosia SV, **Cheng JQ.** Interaction of Phosphatidylinositol-3-OH Kinase/Akt Pathway and Androgen Receptor. *Submitted to JBC*

Dan HC, Coppola D, Jinag K, Liu A, Hamilton AD, Nicosia SV, Sebti SM, **Cheng JQ.** Inhibition of Phosphatidylinositol-3-OH Kinase/Akt and Survivin Pathways by Geranylgeranyltransferase I Inhibitor-298 Induces Apoptosis in Human Ovarian Cancer Cells. *Oncogene, Accepted*

Naidu KA, Yuan ZQ, Sun M, **Cheng JQ,** Coppola D. IGF1 receptor induction of aggressive phenotype in colon carcinoma through PI3K/Akt pathway. *Cancer Res.,* In press

Yuan Z, Feldman RI, Sussman GE, Coppola D, Nicosia SV, **Cheng JQ.** AKT2 inhibition of cisplatin-induced JNK/p38 and Bax activation by phosphorylation of ASK1: Implication of AKT2 in chemoresistance. *J. Biol. Chem.* 278:23432-13440, 2003.

Gritsko TM, Coppola D, Paciga JE, Yang L, Sun M, Shelley SA, Fiorica JV, Nicosia SV, **Cheng JQ.** Activation and Overexpression of Centrosome Kinase BTAK/Aurora-A in Human Ovarian Cancer. *Clin Cancer Res.* 9:1420-1426, 2003.

Cheng J.Q., Jiang X., Fraser M., Li M., Dan H.C., Sun M., Tsang B.K. Role of X-linked Inhibitor of Apoptosis Protein in chemoresistance in Ovarian Cancer: Possible involvement of the PI 3-Kinase/Akt Pathway. Drug Resist Updat. 5:131-146, 2002.

Dan H.C., Sun M., Yang L., Feldman R.I., Sui X-M., Yeung R.S., Halley D.J.J., Nicosia S.V., Pledger W.J., **Cheng, J.Q.** PI3K/AKT pathway regulates TSC tumor suppressor complex by phosphorylation of tuberin. *J. Biol. Chem.* 277:35364-35370, 2002.

Yuan Z., Feldman R.I, Sun M, Olashaw N.E., Coppola D., Sussman G .E., Shelley S.A, Nicosia, S.V. **Cheng, J.Q.** Inhibition of JNK by Cellular Stress- and TNFα-induced AKT2 through Activation of the NFκB Pathway in Human Epithelial Cells. *J. Biol. Chem.* 277:29973-29982, 2002.

Kaneko S, Feldman R.I, Yu L, Wu Z, Gritsko T, Shelley S.A, Nicosia S.V, Nobori T, **Cheng J.Q.** Positive feedback regulation between Akt2 and MyoD during muscle differentiation: Cloning of Akt2 promoter. *J. Biol. Chem.* 277:23230-23235, 2002

Alfonso-De Matte M.Y., Yang H., Evans M.S., **Cheng J.Q.,** Kruk P.A. Telomerase is regulated by JNK in ovarian surface epithelial cells. *Cancer Res.,* 62:4575-4578, 2002.

Sun M, Paciga, J.E, Feldman RI, Yuan Z, Shelley SA, Nicosia SV, **Cheng J.Q.** Phosphatidylinositol-3-OH kinase (PI3K)/AKT2, activated in breast cancer, regulates and is induced by estrogen receptor α (ERα) via interaction between ERα and PI3K. *Cancer Res.,* 61:5985-5991, 2001.

Sun M, Wang, G, Paciga, JE, Feldman RI, Yuan Z, Ma X, Shelley SA, Jove R, Tsichlis PN, Nicosia SV, **Cheng J.Q.** AKT1/PKBα kinase is frequently elevated in human cancers and its constitutive activation is required for oncogenic transformation in NIH3T3 cells. *Am. J. Pathology* 159:431-437, 2001.

Alfonso-De Matte, M.Y, **Cheng, J.Q.,** and Kruk, P.A. Ultraviolet irradiation- and dimethyl sulfoxide-induced telomerase activity in ovarian epithelial cell lines. Exp. Cell Res. 267:13-27, 2001.

Jiang, K., Coppola, D., Crespo, N.C., Nicosia, S.V., Hamilton, A.D., Sebti, S.M. and **Cheng, J.Q.** The phosphoinositide 3-OH kinase/AKT2 pathway as a critical target for farnesyltransferase inhibitor-induced apoptosis. *Mol. Cell. Biol.,* 20:139-148, 2000.

Burdick J. S., Chung E., Tanner G., Sun M., Paciga J. E., **Cheng J. Q.,** Washington K., Goldenring J. R., and Coffey R. J. Monoclonal antibody blockade of the epidermal growth factor receptor improves clinical and biochemical parameters of Menetrier's disease. *The New England J. Med.* 343:1697-1701, 2000.

Yuan, Z., Sun, M., Feldman, R.I., Wang, G., Ma, X., Coppola, D., Nicosia, S.V. and **Cheng, J.Q.** Frequent activation of AKT2 and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/Akt pathway in human ovarian cancer. *Oncogene* 19:2324-2330, 2000.

Kubo, A., Nakagawa, K., Varma, R.K., Conrad, N.K., **Cheng, J.Q.,** Lee, W-C., Testa, J.R., Johnson, B.E., Kaye, F.J. and Kelley, M.J. The p16 status of tumor cell lines identifies small molecule inhibitors specific for cyclin-dependent kinase 4. *Clin Cancer Res.,* 5:4279-4286, 1999.

Cheng, J.Q., Lee, W.-C. Klein, M.A., Cheng, G.Z., Jhanwar, S.C. and Testa, J.R. Frequent alterations of the *NF2* and allelic loss from chromosome 22q12 in malignant mesothelioma: evidence for a two-hits mechanism of *NF2* inactivation. *Genes Chrom. Cancer,* 24:238-242,1999.

Liu, A-X., Testa, J.R., Hamilton, T.C., Jove, R., Nicosia, S.V. and **Cheng, J.Q.** AKT2, a member of the protein kinase B family, is activated by growth factors, v-Ha-ras, and v-src through phosphatidylinositol 3-kinase in human ovarian epithelial cancer cells. *Cancer Res.,* 58:2973-2977, 1998.

Altomare , D.A., Lyons, G.E., Mitsuuchi, Y., **Cheng, J.Q.** and Testa, J.R. Akt2 mRNA is highly expressed in embryonic brown fat and the AKT2 kinase is activated by insulin. *Oncogene* 16:2407-2411,1998.

Ruggeri, B., Huang, L., Wood, M., **Cheng, J.Q.** and Testa, J.R. Amplification and overexpression of the *AKT2* oncogene in a subset of human pancreatic ductal adenocarcinoma. Molecular Carcinogenesis. 21:81-86,1998

Cheng, J.Q., Altomare, D.A., Klein, W.M., Lee, W-C., Kruh, G.D., Lissy, N.A., and Testa J.R. Transforming activity and mitosis-dependent expression of the *AKT2* oncogene: evidence suggesting a link between cell cycle regulation and oncogenesis. *Oncogene* 14:2793-2801, 1997.

Testa, J.R., Liu, Z., Feder, M., Bell, D.W., Balsara, B., **Cheng, J.Q.** and Taguchi, T. Advances in the analysis of chromosome alterations in human lung carcinomas. *Cancer Genet. Cytogenet* 95:20-32, 1997.

Cheng, J.Q., Ruggeri, B., Klein, W.M., Sonoda, G., Altomare, D.A., Watson, D.K. and Testa J.R. Amplification of *AKT2* in human pancreatic cancer cells and inhibition of *AKT2* expression and tumorigenicity by antisense RNA. *Proc. Natl. Acad. Sci. USA* 93:3636-3641, 1996.

Lu Y, Gao C, Cui J, Sun M, **Cheng J.Q.** Deletion and down-regulation of mts1/p16 gene in human gastric cancer. Chinese Journal of Oncology 18(3):189-191, 1996.

Bell, D.W., Yeung, R.S., Bohlander, S.K., **Cheng, J.Q.,** Jin, F. and Testa, J.R. A microdissection library of the rat renal carcinoma gene region. *Cytogenetics and Cell Genetics*, 70:92-94, 1995.

Altomare, D.A., Guo, K., **Cheng, J.Q.,** Sonoda, G., Walsh, K., and Testa J.R. Cloning, chromosomal location, and expression analysis of the mouse *Akt2* oncogene. *Oncogene*, 11:1055-1060, 1995.

Bellacosa, A., Feo, D., Godwin, A. K., Bell, D. W., **Cheng, J. Q.,** Altomare, D., Wan, M., Dubeau, L., Scambia, G., Masciullo, V., Ferrandina, G., Panici P. B., Mancuso, S., Neri, G. and Testa, J. R. Molecular alteration of the *AKT2* oncogene in ovarian and breast carcinomas *Int. J. Cancer*, 64:280-285, 1995.

Bianchi, A.B., Mitsunaga, S-I., **Cheng, J.Q.,** Klein, W.M., Jhanwar, S.C., Seizinger, B., Kley, N., Klein-Szanto, A.J.P., and Testa, J.R. High frequency of inactivations in the neurofibromatosis type 2 (*NF2*) gene in sporadic human malignant mesothelioma. *Proc. Natl. Acad. Sci. USA* 92:10854-10858, 1995.

Cheng, J.Q., Jhanwar, S.C., Klein, W.M., Bell, D.W., Lee, W.-C., Altomare, D.A., Nobori, T., Olopade, O.I., Buckler, A.J. and Testa, J.R. *p16* alterations and deletion mapping of 9p21-p22 in malignant mesothelioma. *Cancer Res.*, 54: 5547-5551, 1994.

Lu, Y.Y., Jhanwar, S.C., **Cheng, J.Q.,** Testa, J.R. Deletion mapping of the short arm of chromosome 3 in human malignant mesothelioma. *Genes Chrom. Cancer* 9:76-80, 1994.

Cheng, J.Q. Molecular cloning and characterization of the *AKT2* oncogene; Genetic alterations in lung cancer and malignant mesothelioma. Paris-XIII University, 1994.

Bellacosa, A., Franke, T.F., Gonzalez-Portal, M.E., Datta, K., Taguchi, T., Gardner, J., **Cheng, J.Q.,** Testa, J.R., Tsichlis, P.N. Structure, expression and chromosomal mapping of c-*akt*: relationship to v-*akt* and its implications. *Oncogene* 8:745-754, 1993.

Cheng, J.Q., Jhanwar, S.C., Lu, Y.Y., Testa, J.R. Homozygous deletions within 9p21-p22 identify a small critical region of chromosomal loss in human malignant mesotheliomas. *Cancer Res.* 53:4761-4764, 1993.

Cheng, J.Q., Godwin, A.K., Bellacosa, A., Taguchi,T., Franke, T.F., Hamilton, T.C., Tsichlis, P.N., Testa, J.R. *AKT2*, a putative oncogene encoding a member of a novel subfamily of serine-threonine protein kinases, is amplified in human ovarian carcinomas. *Proc. Natl. Acad. Sci. USA* 89:9267-9271, 1992.

Watson, D.K., Smyth, F.E., Thompson, D.M., **Cheng, J.Q.,** Testa, J.R., Papas, T.S., Seth, A. The *ERGB/Fli-1* gene: Isolation and characterization of a new member of the family of human ETS transcription factors. *Cell Growth Diff.* 3:705-713, 1992.

Miura, I., Graziano, S.L., **Cheng, J.Q.,** Doyle, L.A. and Testa, J.R. Chromosome alterations in small cell lung cancer: Frequent alteration of 5q. *Cancer Res.* 52:1322-1328, 1992.

Cheng, J.Q., Crepin, M. and Hamelin, R. Pertes d'alleles sur les chromosomes 1p, 3p, 11p et 11q dans les cancers du poumon non a petites cellules. *C.R. Acad. Sci. Paris* 311:175-180, 1990.

Cheng, J.Q., Crepin, M. and Hamelin, R. Loss of heterozygosity on chromosomes 1p, 3p, 11p and 11q in human non-small cell lung cancer. In: Papillomaviruses in Human Pathology. Recent Progress in Epidermoid Precancers, J. Mousenego (ed.). Serono Symposia Publications from Raven Press, New York, Vol. 78, 357-364, 1990.

Zhao, Z.W., Xiu, S.F. and **Cheng, J.Q.** Bilateral bronchoplasty to treat multiple bronchial tumors. *Chinese J. Surg.* 27:278, 1989.

Zhao, Z.W, Li, S.Y., Fu, Y. and **Cheng, J.Q.** Pulmonary artery reconstruction and bronchoplastic procedure in the treatment of lung cancer. *Chinese J. Surg.* 26:176-178, 1988.

Cheng, J.Q., Wang, T.R, Li, H. and Zhang, S.S. Thin needle biopsy of pulmonary lesions: report of 90 cases. *Chinese J. Thorac Cardiovasc Surg.* 2:87-89, 1986.

Wang, T.R. and **Cheng, J.Q.** Effects of omentumectomy on alimentary tract perforation and abdominal cavity infection: an experimental observation. *J. North China Med.* 2:50-54, 1986.

Cheng, J.Q., Wang, T.R and Zhang, S.S. Carcinomatous lung abscess. *North China Med.* 5:321-323, 1983.

Book Chapter:

Cheng, J.Q. and Nicosia, S.V. PI3K/Akt signal tranduction pathway in oncogenesis. Cancer Research Encyclopedia, 2000.

Yuan, Z-Q and Cheng, J.Q. Akt survival pathway. Molecular and Cellular Oncology, 2001

Abstracts:

Cheng, J.Q., Jhanwar, S.C., Lu, Y.Y., Testa, J.R. Homozygous deletions within 9p21-p22 identify a small critical region of chromosomal loss in human malignant mesotheliomas. Proceedings of American Association for Cancer Research, 34, 1994.

Cheng, J.Q., Jhanwar, S.C., Klein, W.M., Nobori, T., Olopade, O.I. and Testa, J.R. *p16* alterations and deletion mapping of 9p21-p22 in malignant mesothelioma. 86th Annual Meeting of American Association for Cancer Research, Toronto, 1995.

Cheng, J.Q., Altomare, D.A., Klein, W.M., Lee, W-C., Kruh, G.D., Lissy, N.A., and Testa J.R. Transforming activity and cell cycle-dependent expression of AKT2: evidence for cell cycle control and malignant transformation. Proceedings of the American Association for Cancer Research, 38:539, 1997.

Yeung, R.S., Xiao, G.H., Kisielewski, A., Klein-Szanto, A.J.P., Sina, J., Bleicher, K., **Cheng, J.Q.,** Testa, J.R. and Goodrow, T.L. Alteration of *FHIT* protein expression in human neoplasia. Proceedings of the American Association for Cancer Research, 38:2826, 1997.

Jiang, C., Yuan, W., Liu, A-X., Cao, C-H., Jiang, K., Nicosia, S.V. and **Cheng, J.Q**. Alterations of β-catenin Oncogene in Human Ovarian Cancer. Proceedings of the American Association for Cancer Research, 40:2468, 1999.

Jiang, K., Coppola, D., Crespo, N.C., Nicosia, S.V., Hamilton, A.D., Sebti, S.M. and **Cheng, J.Q.** The phosphoinositide 3-OH kinase/AKT2 pathway as a critical target for farnesyltransferase inhibitor-induced apoptosis. 15th Annual Meeting on Oncogenes and Tumor Suppressors NCI, 1999.

Yuan, Z., Sun, M., Feldman, R.I., Wang, G., Ma, X., Coppola, D., Nicosia, S.V. and **Cheng, J.Q.** Frequent activation of Akt and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/Akt pathway in human ovarian cancer. 91th Annual Meeting of American Association for Cancer Research, San Francisco, 2000.

Cheng, J.O., Jiang, K., Coppola, D., Crespo, N.C., Nicosia, S.V., Hamilton, A. D. and Sebti, S.M. AKT2 as a critical target for farnesyltransferase inhibitor-induced apoptosis. 91th Annual Meeting of American Association for Cancer Research, San Francisco, 2000.

Hakam, A., Reed, J., Boulware, D., **Cheng, J.Q.,** Nicosia, S.V., Coppola, D. Modulation of Fas receptor (FasR) expression in benign and malignant epithelial ovarian lesions. 91th Annual Meeting of American Association for Cancer Research, San Francisco, 2000.

Sebti, S. M., Jiang, K., Coppola, D., Crespo, N. C., Nicosia, S.V., Hamilton, A. D. and **Cheng, J.Q.** FTI-277, a farnesyltransferase inhibitor, induces apoptosis by blocking he phosphoinositide 3-OH kinase/AKT2 survival pathway in human cancer cells. 91th Annual Meeting of American Association for Cancer Research, San Francisco, 2000.

Sun, M., Dan, H., Feldman, R.I., Jove, R., Nicosia, S.V., and **Cheng, J.Q.** AKT1/PKBα activity is frequently elevated in human cancers and required for oncogenic transformation; dominant negative Akt inhibits Ras, transformation. Tumor Suppressor Gene, Cold Spring Harbor, 2000.

Yuan, Z-Q., Coppola, D., Olashaw, N.F., Nicosia, S. V., **Cheng, J.Q.** AKT2/PKBβ Is Activated by Cellular Stresses and TNFα through Phosphatidylinositol 3-kinase and Targets NF□B, but not JNK1 or p38 Pathway. 92th Annual Meeting of American Association for Cancer Research, 2001.

Crespo, N.C., Sun, J., Ohkanda, J., Coppola, D., Hamilton, A., **Cheng, J.Q.,** and Sebti, S.M. The farnesyltransferase inhibitor, FTI-2148, inhibits tumor growth and induces apoptosis in vivo in OVCAR3 tumors, but not OVCAR3 tumors overexpressing activated AKT2. 92th Annual Meeting of American Association for Cancer Research, 2001.

Yuan, Z., Kaneko, S., Dan H-C., Nicosia, S.V., and **Cheng, J.Q.** Akt/PKB Binding Protein, APαB, Mediates Akt Survival Signal through Activation p21-activated Kinase. 7th Annual Meeting on Oncogenes: Cancer Cell Signal Transduction, 2001

Kaneko, S., Sun, M., Gritsko, T., Nicosia, S.V., Nobori, T., and **Cheng, J.Q.** AKT2 Is Transcriptionally Regulated by MyoD and Required for MyoD-induced Skeletal Muscle Differentiation. 7th Annual Meeting on Oncogenes: Cancer Cell Signal Transduction, 2001

Sun, M., Paciga, J.E., Feldman, R.I., Yuan, Z., Nicosia, S.V., and **Cheng, J.Q.** AKT2, activated in breast cancer, regulates estrogen receptor a that stimulates PI3K/AKT2 pathway via nongenomic mechanism. 7th Annual Meeting on Oncogenes: Cancer Cell Signal Transduction, 2001.

Dan H.C., Sun M., Yang L., Feldman R.I., Sui X-M., Yeung R.S., Halley D.J.J., Nicosia S.V., Pledger W.J., **Cheng, J.Q.** Interaction of TSC tumor suppressor complex with Akt/PKB pathway. 8th Annual Meeting on Oncogenes: Cancer Cell Signal Transduction, 2002.

Yuan Z., Feldman R.I, Sun M, Nicosia, S.V. **Cheng, J.Q.** AKT interaction proteins and cell survival. Apoptosis and Human Cancer, AACR, 2002.

Cheng, J.Q. Fiorica, S.V. Nicosia, S.V. BTAK and PI3K/Akt pathways in human ovarian cancer. Third Annual International Conference on Ovarian Cancer, 2002.

Yuan Z-Q., Kaneko S., Coppola D., Nicosia S.V. **Cheng, J.Q.,** APαB mediates Akt survival signal: implication in chemoresistance in breast cancer. DOD Annual Breast Cancer Conference, 2002.

Yang, H., Paciga, J.E., Coppola, D., Shelley, S.A., Nicosia, S.V., **Cheng, J.Q.** PI3K/AKT2 Pathway mediates Her2/Neu-induced telomerase activation in human breast cancer cells. DOD Annual Breast Cancer Conference, 2002.

Yuan Z, Feldman RI, Sussman GE, Coppola D, Nicosia SV, Cheng JQ. AKT2 Inhibition of Cisplatin-induced JNK/p38 and Bax Activation by Phosphorylation of ASK1: Implication of AKT2 in Chemoresistance. 94th American Association for Cancer Research Annual Meeting, 2203.

Gritsko TM, Coppola D, Paciga JE, Yang L, Sun M, Shelley SA, Fiorica JV, Nicosia SV, Cheng JQ. Alterations of Aurora-A oncogene in human ovarian cancer. 94th American Association for Cancer Research Annual Meeting, 2203.

Yuan Z, Kaneko S, Coppola D, Nicosia SV, Cheng JQ. Akt-interacting protein, APαB, mediates PI3K/Akt survival signal through activation of PAK1 and NFκB pathways. 94th American Association for Cancer Research Annual Meeting, 2203.

Kaneko S, Yang L, Paciga J, Yu H, Nicosia SV, Jove R, Cheng JQ. Transcriptional Upregulation of AKT2 by Stat3 and Src. 19th Annual Meeting on Oncogene, 2003.

Dan DC, Sun M, Feldman RI, Nicosia SV, Wang H-G, Tsang B, Cheng, JQ. Akt Regulates X-linked inhibitor of apoptosis, XIAP: A Mechanism to Antagonize Cisplatin-Induced Apoptosis in Human Ovarian Epithelial Cancer Cells. 19th Annual Meeting on Oncogene, 2003.

Sun M, Yuan Z, Yang L, Feldman RI, Yeatman TJ, Jove R, Nicosia SV, Cheng JQ. AKT2 Inducible Gene hHb1-ΔN Feedback Activates PI3K/Akt Pathway and Promotes Cell Proliferation and Cell Survival. 19th Annual Meeting on Oncogene, 2003.

Yang L, Sun M, Yang H, Dan HC, Nicosia SV, Cheng JQ. Akt/PKB Inhibits HtrA2/Omi-stimulated Apoptosis through Direct Disruption of its Serine Protease Activity. 19th Annual Meeting on Oncogene, 2003.

Yuan Z, Feldman RI, Sussman GE, Coppola D, Nicosia SV, Cheng JQ. ASK1 is a target to overcome Akt induced cisplatin resistance. 19th Annual Meeting on Oncogene, 2003.

Cheng JQ. Akt and prenylation inhibitors as potential reagents to overcome p53-assocated chemoresistance in human ovarian cancer. The Gynecologic Oncology Group Meeting, 2003.

Invited Presentations:

Akt and prenylation inhibitors as potential reagents to overcome p53-assocated chemoresistance in human ovarian cancer. The Gynecologic Oncology Group Meeting, 2003.

PI3K/Akt as critical target for cancer intervention. American Society of Clinical Oncology, Molecular Therapeutics Symposium. San Diego, California, 2002

Role of the Akt oncogene in human ovarian cancer. Third Annual International Conference on Ovarian Cancer, Houston, Texas, 2002

PI3K/AKT pathway in Cell Survival, Cell Cycle and Differentiation. M.D. Anderson Cancer Center, Houston TX, April 25, 2002

AKT pathway and human oncogenesis. M.D. Anderson Cancer Center, Houston TX, January 6, 2002

Phosphatidylinositol-3-OH kinase/Akt pathway and cell survival. The Joy McCann Culverhouse Airway Disease Center, USF, Florida, 2001

PI3K/AKT pathway and human malignancy. University of Ottawa, Ontario, Canada, 2001.

Akt/PKB as a critical target for cancer prevention. Berlex Bioscience, Richmond, California, 1999.

PI3K/Akt pathway in human cancer. Berlex Bioscience, Richmond, California, 1998.

Cloning strategies of oncogenes and tumor suppressor genes. Beijing Institute for Cancer Research, Beijing, China, 1998.

PI3K/Akt pathway in cell cycle and apoptosis. Chinese Academy of Medical Science and Peking Union Medical College, Beijing, China, 1998

Tumor suppressors p16 and NF2 in human malignancy. Tianjin Medical School, Tianjin, China, 1998.

Akt survival pathway in human malignancy. Beijing University, Beijing, China, 1998.

Platform Presentations:

Yang L, Sun M, Yang H, Dan HC, Nicosia SV, **Cheng JQ**. Akt/PKB Inhibits HtrA2/Omi-stimulated Apoptosis through Direct Disruption of its Serine Protease Activity. 19th Annual Meeting on Oncogene, 2003.

Yuan, Z., Kaneko, S., Dan H-C., Nicosia, S.V., and **Cheng, J.Q.** Akt/PKB Binding Protein, APαB, Mediates Akt Survival Signal through Activation p21-activated Kinase. Oncogene Meeting, 2001

Jiang, K., Coppola, D., Crespo, N.C., Nicosia, S.V., Hamilton, A.D., Sebti, S.M. and **Cheng, J.Q.** The phosphoinositide 3-OH kinase/AKT2 pathway as a critical target for farnesyltransferase inhibitor-induced apoptosis. 15th Annual Meeting on Oncogenes and Tumor Suppressors. National Cancer Institute, 1999.

Cheng, J.Q., Lee, W.-C. and Testa, J.R. Frequent mutation of the *NF2* gene and deletion of chromosome 22q13 in malignant mesothelioma: evidence of two-hits of NF2 inactivation. Conference of International Association for the Study of Malignant Mesothelioma, Philadelphia, 1997.

Cheng, J.Q., Jhanwar, S.C., Klein, W.M., Nobori, T. and Testa, J.R. *p16* alterations in malignant mesothelioma. Conference of International Association for the Study of Lung Cancer, Colorado Springs, 1994.

Cheng, J.Q., Ruggeri, B., Klein, W.M, Altomare, D.A. and Testa J.R. Amplification of *AKT2* in human pancreatic cancer cells and inhibition of *AKT2* expression and tumorigenicity by antisense RNA. 85th Annual Meeting of American Association for Cancer Research, San Francisco, 1994.

Cheng, J.Q. Cai, L.P. Wang, T.R. Zhang, S.S. and Xin, Y.L. Effects of omentumectomy on alimentary tract perforation and abdominal cavity infection: an experimental observation. International Thoracic Surgery Conference, Beijing, 1986.

Predoctoral and Postdoctoral Trainees:

Ph.D.:

1998-2002 Zengqiang Yuan, Graduate student,
(obtained three years' Predoctoral Fellowship from Department of Defense)

1999-present Mei Sun, Postdoctoral
(submitted Postdoctoral Fellowship to Department of Defense in 2001.6)
Satoshi Kaneko, Postdoctoral

2000-present Hua Yang, Research Associate
Tanya Gritsko, Postdoctoral
Hansso Lee, Postdoctoral
Lin Yang, Postdoctoral
Han-cai Dan, Graduate student
Grace Sui, Graduate student

2001-present June Paciga Research Associate
Donghwa Kim Postdoctoral
Sungman Park Graduate student
Kirk Townsend Graduate student

1997 Ai-xie Liu, Postdoctoral
(finished 1998, now Research Associate at University of Pennsylvania)

1998 Gen Wang, Postdoctoral
(finished 2000, now Research Associate at University of British Columbia, Canada)
Kun Jiang, Postdoctoral

(finished 1999, now Research Associate at USF).
Chuan H. Cao, Postdoctoral
(finished 1999, now postodoctoral at USF).

M.D.:

1995-1996 Edward R. Sauter (Now Assistant Professor at Thomas Jefferson University, Philadelphia)

1999-2000 Xiao-ling Mao (Due to her accomplishment in my laboratory, she was awarded in Pathology Annual Research Conference. Now Fellowship at University of Texas).

2001 Marianna Szabo (Now at Moffitt Cancer Center)

Undergraduate and high school student:

1999 Jannifer Chang (Boston University, MA)
2000 Teffini (University of South Florida, Tampa, FL)
Gene Susman (Long Island High School, New York)
2001 Jason Ou (Rice University, Houston, TX),
Gene Susman (Long Island High School, New York)
Ellen Sun (Whaton High School, Tampa, FL)

Ph.D. Thesis Committees:

1998-2000: Todd Samelman, (Graduated in May 2000, now student at University of North Carolina)
1998-2001: Hongqian Zhang, (Graduated in March 2001, now postdoctoral at H. Lee Moffitt Cancer Center)
1998-2002 Michelle Alfonso-De Matte, graduate student at Department of Pathology, USF
1999-present Wilson Yuan, graduate student at Department of Pathology, USF
2001-present Caroline Desponts, graduate student at Department of Biochemistry and Molecular Biology, USF
2001-present Nicole Johnson, graduate student at Department of Pathology, USF
2002-present Feng Jiang, graduate student at Department of Pathology, USF
2002-present Han C. Dan, graduate student at Department of Pathology, USF
2002-present Kirk Townsend, graduate student at Department of Pathology, USF
2002-present Sungman Park, graduate student at Department of Pathology, USF

Teaching Activities:

1998-present Lectures for graduate student seminar series.

1998-present I have had four graduate students and 10 postdoctoral fellows during this time. One graduate student obtained a three-year Fellowship from the Department of Defense. Three postdoctoral fellows and 2 graduate students received research and travel awards at different Research Conferences.

2001 Advance Cell Signaling (Spring Semester; The course was opened by myself; Two hours/week; GMS 7930, Section 008, Reference #17924; 2 credits).

2001 Tumor Genetics (Summer Semester; The course was opened by myself; Two hours/week; GMS 7930, Section 003, Ref. #52431, Session B, 3 credits).

2001 Advance Cell Signaling (Fall Semester; The course was opened by myself; Two hours/week; GMS 7930, Section 005, 3 credits).

Editorial Service:

Proceedings of National Academy of Sciences of United States of America
Oncogene
Cancer Research
Molecular Cancer Research
Molecular Cancer Therapeutics
Carcinogenesis
Molecular Carcinogenesis
Biochimica et Bioophysica Acta
Gynecologic Oncology
Physiological Genomics

Professional Societies:

American Association for Advancement of Science
American Association for Cancer Research
American Society for Microbiology
American Society for Biochemistry and Molecular Biology

USF Committee:

As member:
Graduate Council (2001-present)
Research committee (1999-present)
LEMC committee (1998-1999)
Medical School Student Learning communities (2001-present)
Department APT committee (2002-present)
As chair:
Graduate Student Oncology Section USF/HSC Research Day (1998-2002)

Consultant:

Berlex Biosciences, Richmond, CA.